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14. ABSTRACT

Breast cancers in humans often grow slowly or even remain undetectable for long periods of time only to reappear in discreet stages as progressively more malignant tumors. Recently, studies in both human cancers and experimental cancers in animals have established that cancers become progressively more aggressive in incremental steps that result from genetic mutations or "switches" in the tumor cells themselves.

We have found that the two growth/differentiation promoting cytokines pleiotrophin (PTN) and midkine (MK) act as "switches" when introduced into breast cancer cells to stimulate more aggressive growth and induce new intratumor blood vessel formation, ie, an "angiogenic switch." Different studies have found constitutive expression of either the PTN or MK genes in over 50% of human breast cancers, suggesting our data is very important and relevant to human breast cancer. We now plan to pursue the mechanism of PTN signaling in both MMTV driven pleiotrophin gain of function mice and "knock-out" pleiotrophin mice developed in the laboratory and the mechanisms of downstream PIN signaling with different "chip technology"-driven strategies available to us in the laboratory.

15. SUBJECT TERMS
Pleiotrophin, Midkine, Tumor Angiogenesis, Epithelial-Mesenchymal, and Angiogenic Pathways

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Table of Contents

FINAL REPORT, Army Grant DAMD17-00-1-0151

OBJECTIVES OF GRANT

Objective I

The achievement of an appropriate cDNA library from primary human breast cancers as a source of genes potentially able to use PTN to effectively enhance MCF 10A cell growth in the nude mouse.

The development of MCF 10A-PTN,-PTN mutant cell lines.

Objective II

The identification of downstream interactive proteins with the receptor protein tyrosine phosphatase (RPTP)□□and thus of the PTN signaling pathway in human breast cancer cells.

REPORTABLE OUTCOMES

Objective I:

Pleiotrophin (PTN, *Ptn*) was identified in different screens of breast cancers and breast cancer cell lines and more recently, in differential microarray analyses to be expressed in high levels in different breast cancers and to be constitutively expressed in cell lines derived from these breast cancers. Instead of MCF 10A cells, the laboratory chose to test MCF-7 human breast cancer cells and other cell lines, including 3 other breast cancer cell lines and now, MMTV-*PyMT/Ptn* bitransgenic mice. The identification of *Ptn* in these screens has been extremely valuable.

The following studies led to or resulted from Objective 1.

Identification of the Angiogenesis Signaling Domain in Pleiotrophin Defines a Mechanism of The Angiogenic Switch. Nan Zhang, Rong Zhong, Pablo Perez-Pinera, Gonzalo Herradon, Laura Ezquerra, Zhao-Yi Wang and Thomas F. Deuel**.** Biochem. Biophys. Res. Commun 343: 653-658, 2006.

We introduced an ectopic *Ptn* gene into "premalignant" SW-13 cells and analyzed the phenotype of SW-13 *Ptn* cell tumor implants in the flanks of nude mice. Pleiotrophin is shown to stimulate SW-13 *Ptn* cell subcutaneous tumor implants growth (Table 1 of enclosed manuscript) and to stimulate increase in the density of new blood vessels compared to the SW-13 cell tumor implants (Figure 2). The data establishes that constitutive PTN signaling in SW-13 cell implants recapitulates fully the angiogenic switch in the nude mouse. The data also demonstrates that the C-terminal domain of

PTN is the "angiogenesis domain" and makes distinction that the C-terminal and the N-terminal domains of PTN equally initiate switches in premalignant cells to cells of a more aggressive tumor phenotype.

Significance: The significance of these studies is high; the appearance of new blood vessels in malignant tumors is known as the "angiogenic switch". The angiogenic switch triggers a stage of rapid tumor growth supported by extensive tumor angiogenesis and a more aggressive tumor phenotype. Its onset is a poor prognostic indicator for host survival. Thus, identification of the factors that stimulate the angiogenic switch is of high importance.

These studies use a nude mouse model to demonstrate *Ptn* constitutively expressed in SW-13 cells alone is sufficient to trigger an angiogenic switch *in vivo*. Pleiotrophin expression is constitutive in many human tumors; the study thus identifies the ability of PTN to stimulate tumor angiogenesis *in vivo*. The study thus complements the studies that demonstrate that PTN stimulates angiogenesis above. The study suggests a mechanism that PTN through activation of angiotensin II signaling PTN stimulates new blood vessels but furthermore stimulates the collagen and elastin extracellular matrix needed for new vessel formation.

Pleiotrophin Induces Formation of Functional Neovasculature *in vivo*. Christman, K.L., Fang, Q., Kim, A. J., Sievers, R.E., Fok, H.H., Candia, A.F., Colley, K.J., Ezquerra, L., Herradon G., Deuel, T.F., Lee, R.J. (2005). Biochem. Biophys. Res. Commun. 332 (4) 1146-1152.

Pleiotrophin is shown to be directly angiogenic; PTN injected into ischemic myocardium stimulated significant increases in both normal appearing new capillaries and arterioles and the newly formed blood vessels interconnect with the existent coronary vascular system (50).

Significance: The significance of this study is the direct demonstration that PTN is an effective angiogenic agent *in vivo;* it is demonstrated that PTN initiates new capillary and arterioles formation that is both normal in appearance and functional in blood flow. It is compatible with Studies above that led us to identify that PTN upregulates the renin-angiotensin pathway, and through stimulation of angiotensin II signaling, PTN stimulates new blood vessel formation and the collagen and elastin needed in support of the vessels.

Constitutive Expression Of Rb Associated Protein 46 (RbAp46) Reverts Transformed Phenotypes Of Breast Cancer Cells. Zhang TF, Yu SQ, Deuel TF, Wang ZY. (2003). Anticancer Research 23: 3735-3740.

Significance: The data demonstrate that constitutive expression of RbAp46 suppresses the transformed phenotypes of breast cancer cells. The significance of this study is high, since Rbp46 is identified to have a tumor suppressor function in breast cancer cells.

Identification, Cloning, And Expression Of Human Estrogen Receptor-36, A Novel Variant Of Human Estrogen Receptor-66. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. (2005). Biochem Biophys Res Commun 336 (4) 1023-7.

Significance: The identification and subsequent cloning of the 36kDa human estrogen receptor (here termed hER-36) maybe very important; it supports the hypothesis that alternative signaling pathways are found in human breast cancer that were previously unsuspected.

Studies in Process of Publication Derivative from the Data supported by Objective 1:

An MMTV-Pleiotrophin Transgene Promotes More Aggressive Growth and Induces a Morphological Phenotype of Invasive Ductal Carcinoma with Increased Tumor Angiogenesis in breast Cancers of MMTV-Polyoma Middle T (PyMT) Mice

To test whether PTN functions as an oncogenic protein to induce breast cancer or as a promoter of a more malignant phenotype during breast cancer progression, transgenic mice that express the mouse mammary tumor virus (MMTV) promoter-driven *Ptn* gene (MMTV-*Ptn*) were generated; MMTV-*Ptn* single transgenic mice failed to develop breast cancers, thus, inappropriate *Ptn* expression alone is not sufficient to induce breast cancer in these mice. MMTV-*Ptn* single transgenic mice were then bred with MMTV-*PyMT* (Polyoma Middle T antigen) single transgenic mice; both MMTV-*PyMT*-*Ptn* bi-transgenic mice and MMTV-*PyMT* single transgenic mice rapidly developed multi-focal breast cancers; the average size of the breast cancers in MMTV-*PyMT*-*Ptn* bi-transgenic mice was ~ 2-fold increased relative to MMTV-*PyMT* single transgenic mice and contained an ~ 2-fold increase of foci that morphologically are strikingly similar to the scirrhous patterned invasive ductal carcinomas in humans; in every breast cancer examined, these foci expressed very high levels of the MMTV-*Ptn* transgene. They were surrounded circumferentially by striking increases in collagen and elastin interspersed with “carcinoma associated fibroblast” (CAF)-like fibroblasts and new blood vessels of

significantly larger size. In MMTV-*PyMT-Ptn* breast cancers, levels of activated ERK1/2 were increased and the levels of expression of the 46 kD mouse estrogen receptor isoform were increased. It is concluded that pathways activated by PTN signaling cooperate with pathways activated by constitutive PyMT signaling to "switch" MMTV-*PyMT* mouse breast cancers to a more aggressive phenotype resembling scirrhous patterned invasive ductal carcinomas in human breast cancers. Furthermore, properties of MMTV-*PyMT-Ptn* breast cancers of increased collagen and elastin synthesis and new blood vessels correspond directly with previously described properties of PTN-stimulated cells and cells with constitutive expression of *Ptn*, suggesting the very high level expression of MMTV-*Ptn* transgene in breast carcinoma of MMTV-*PyMT-Ptn* mice resembling scirrhous patterned invasive ductal carcinoma underlies the mechanism that drives the development of MMTV-*PyMT-Ptn* bi-transgenic mouse breast cancers.

Secretion of Pleiotrophin from MCF-7 Human Breast Cancer Cells Activates Stromal Fibroblasts, Induces Sharply Circumscribed MCF-7 Cell Epithelial Islands, and Remodels the Cancer Cell Microenvironment: A Model of Tumor Progression

Activation of stromal fibroblasts by factors released from carcinoma cells underly the mechanisms of tumor progression and remodeling of the microenvironment. Pleiotrphin (PTN, *Ptn*) is an 18 kD secreted cytokine expressed in high levels in many breast cancers and other cancers of epithelial origins and its endogenous gene is inappropriately expressed in cell lines derived from these cancers. Previous studies furthermore demonstrate that aboragation of PTN-signaling through expression of dominant negative PTN reversed the malignant phenotype of these cells. To test the contribution of PTN signaling in the breast cancer cells, we injected MCF-7 cells that express an exogenous *Ptn* gene (MCF-7-*Ptn* cells) in the flanks of nude mice. MCF-7 cells do not express the endogenous *Ptn* gene nor do they express the PTN receptor, the Receptor Protein Tyrosine Phosphatase (RPTP)□/□, and thus, MCF-7-*Ptn* cells are not responsive to PTN through paracrine stimulation. The MCF-7-*Ptn* cell xenografts grew rapidly; their rate of growth was even more rapid if co-injected with equal numbers of NIH3T3 cells, but MCF-7 cell xenografts were barely detectable 6 weeks after injection. Surprisingly, sections stained with hematoxylin and eosin revealed that the MCF-7-*Ptn* cell explants developed a morphological phenotype that closely resembles human invasive ductal carcinoma. To better understand the significance of these findings, MCF-

7-*Ptn* cells were co-cultured with NIH 3T3 cells; it was found that secretion of PTN from MCF-7-*Ptn* cells induced formation of sharply defined clusters of MCF-7-*Ptn* cells, previously described as "epithelial islands", consisting of less well differentiated MCF-7-*Ptn* cells that were not restricted by cell-cell contact and surrounded by dense fibrous bands containing abundant tropoelastin and NIH3T3 cells that morphologically resembled “carcinoma associated fibroblasts” (CAFs). Expression of tropoelastin and type IV procollagen was markedly increased in the CAF-like cells and activated protein kinase C (PKC)-□ was found in MCF-7-*Ptn* and the CAF-like cells and both human and murine matrix metalloproteinase (MMP) 9 was readily identified in media of co-cultures. The striking biochemical and morphological features in co-cultures are *Ptn* expression dependent, PTN-secretion dependent, and co-culture dependent. The data suggest that PTN secretion alone from human breast cancer cells alone initiates reciprocal signaling between the carcinoma stromal cells and the PTN secreting breast cancer cells and induces up-regulation of new collagen and elastin synthesis and PKC□ and MMP9 and extensive remodeling of the microenvironment, and a morphological transition of the MCF-7-*Ptn* cells and NIH3T3 cells to phenotypes commonly seen in human breast carcinomas.

Objective II

The following studies led to or resulted from Objective 1. The identification of RPTP □/□ as the physiological receptor of PTN has led to the identification of many downstream signaling molecules in the PTN/RPTP □/□ signaling pathway.

Pleiotrophin Stimulates Tyrosine Phosphorylation of □-Adducin Through Inactivation of The Transmembrane Receptor Protein Tyrosine Phosphatase (RPTP)□/□. Pariser, H., Perez-Pinera, P., Ezquerra, L., Herradon, G., Deuel, T.F (2005), Biochem Biophys Res Commun. 335 (1) 232-9.

This study showed that □-adducin a substrate of RPTP□/□; it is dephosphorylated by RPTP□/□ and its levels of tyrosine phosphorylation are increased in PTN-stimulated cells. Beta-adducin is the second downstream target of the PTN/RPTP□/□ signaling pathway to be identified (22); □-adducin belongs to a family of proteins that bind to actin-spectrin junctions and stabilize the growing actin filaments and actin-spectrin networks (47-49).

Pleiotrophin Regulates Serine Phosphorylation and the Cellular Re-Distribution of β-Adducin Through Activation of Protein Kinase C. Pariser H, Herradon G, Ezquerra L, Perez-Pinera P, Deuel TF. (2005). Proc Natl Acad Sci U S A. 102 (35) 12407-12.

Our studies show that PTN activates protein kinase C (PKC) and stimulates the PTN-dependent PKC-catalyzed phosphorylation of serines 713, 726 of β-adducin. Phosphorylation of serines 713, 726 in β-adducin markedly reduces the affinity of β-adducin for spectrin and actin and uncouples actin/spectrin/β-adducin multimeric complexes needed to stabilize the cytoskeleton; the PTN stimulated phosphorylation of serines 713, 726 in β-adducin thus contributes to the disruption of cytoskeletal complexes. PTN also stimulates translocation of β-adducin phosphorylated in serines 713, 726 to either nuclei where it is associated with nuclear chromatin and centrioles of dividing cells or to a membrane associated site, depending on the phase of cell growth.

Significance: This data demonstrates that PTN regulates cytoskeletal stability. Pleiotrophin regulates the cellular location of β-adducin. The discovery of β-adducin in nucleus is a unique finding; the study thus demonstrates PTN disrupts cytoskeletal protein complexes and integrity and determines the sites in the cells where β-adducin is localized; it suggests β-adducin may function potentially in the nucleus in support of heterochromatin structure and centrioles during mitosis.

Fyn is a Downstream Target of The Pleiotrophin/Receptor Protein Tyrosine Phosphatase (RPTP)β/ζ Signaling Pathway: Regulation of Tyrosine Phosphorylation of Fyn by Pleiotrophin. Pariser H, Ezquerra L, Herradon G, Perez-Pinera P, Deuel TF (2005). Biochem. Biophys. Res. Commun. 332 (3) 664-9.

It was demonstrated that Fyn binds to the active site (D1) domain of RPTPβ/ζ, that Fyn is a substrate of RPTPβ/ζ, and that tyrosine phosphorylation of Fyn is sharply increased in PTN-stimulated cells (24). Fyn is a Src-like kinase. Subsequently it was shown (see Specific Aim 2) that Fyn phosphorylates tyrosines in proteins that are substrates of RPTPβ/ζ and, Fyn kinase is inactivated in PTN-stimulated cells.

Significance: The data demonstrate that Fyn is a downstream target of the PTN/RPTPβ/ζ signaling pathway; it is later shown that Fyn kinase is inactivated in PTN-stimulated cells and thus its activity is regulated by PTN in PTN-stimulated cells.

Pleiotrophin is an important regulator of the renin–angiotensin system in mouse Herradon G, Ezquerra L, Nguyen T, Vogt TF, Bronson R, Silos-Santiago I, Deuel TF. (2004). Biochem. Biophys. Res. Commun. 324 (3) 1041-1047.

Transcriptional profiling studies in *Ptn -/-* mice uncovered a striking downregulation of each of the genes encoding both the enzymes and the angiotensin type 1 and 2 receptors of the renin-angiotensin pathway.

Significance: As shown subsequently, these studies are of major importance; they have led to the demonstration that angiotensin II downstream signaled pathways in human cancer cells that express the *Ptn* gene stimulate new collagen, new elastin, and new blood vessel growth that is both PTN and angiotensin II-dependent. These data demonstrate for the first time the levels of *Ptn* expression regulate levels of expression of the genes encoding the key proteins of the renin–angiotensin system in mouse; the data support the hypothesis that levels of *Ptn* gene expression critically regulate angiogenesis, collagen synthesis, and elastin synthesis. This study led to the discovery that increased angiogenesis associated with *Ptn* expression in tumors is reversed with a clinically tested inhibitor of the renin-angiotensin system *in vivo* and led us to now test gain of function PTN in breast cancer predisposition mice with ACE inhibitors to seek to reverse angiogenesis and new collagen and new elastin synthesis. It has opened an important area of research in PTN signaling. It explains in part how PTN through this downstream signaling pathway remodels extracellular matrix and regulates new blood vessel formation.

Midkine, a Newly Discovered Regulator of the Renin–Angiotensin Pathway in Mouse: Significance of the Pleiotrophin/Midkine Developmental Gene Family in Angiotensin II Signaling. Ezquerra L, Herradon G, Nguyen T, Silos-Santiago I, Deuel TF, (2005) Biochem. Biophys. Res. Comm. 333 (2) 636-643.

Significance: The data supported MK and PTN share striking but not complete functional redundancy and made clear that MK regulates levels of downstream signals initiated by angiotensin II. The functional overlap is important, and, since MK is upregulated in new blood vessels, this study identifies a site of functional overlap and supports strongly the role of MK in stimulation of new blood vessel formation at sites of injury as cited above with PTN.

Midkine Regulates Pleiotrophin Organ-Specific Gene Expression: Evidence For Transcriptional Regulation and Functional Redundancy Within The Pleiotrophin/Midkine Developmental Gene Family. Herradon G, Ezquerra L, Nguyen T, Silos-Santiago I, Deuel TF (2005) Biochem. Biophys. Res. Commun. 333 (3) 714-21.

Significance: The data demonstrate for the first time that MK regulates *Ptn* gene expression with a high degree of organ specificity. This study supports the functional

overlap in the PTN-MK developmental gene family and complements earlier studies to focus on overlap in new collagen synthesis and new blood vessel formation that are mediated through the renin-angiotensin signaling pathway.

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Pleiotrophin is an important regulator of the renin–angiotensin system in mouse aorta

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Abstract

To better understand the phenotype of pleiotrophin (PTN the protein, *Ptn* the gene) genetically deficient mice (*Ptn* −/−), we compared the transcriptional profiles of aortae obtained from *Ptn* −/− and wild type (WT, *Ptn* +/+) mice using a 14,400 gene microarray chip (Affymetrix) and confirmed the analysis of relevant genes by real time RT-PCR. We found striking alterations in expression levels of different genes of the renin–angiotensin system of *Ptn* −/− mice relative to WT (*Ptn* +/+) mice. The mRNA levels of the angiotensin converting enzyme (ACE) were significantly decreased in *Ptn* −/− mice whereas the mRNA levels of the angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors were significantly increased in *Ptn* −/− mice when they were compared with mRNA levels in WT (*Ptn* +/+) mice aortae. These data demonstrate for the first time that the levels of expression of the *Ptn* gene markedly influence expression levels of the genes encoding the key proteins of the renin–angiotensin system in mouse aorta and suggest the tentative conclusion that levels of *Ptn* gene expression have the potential to critically regulate the downstream activities of angiotensin II, through the regulation of its synthesis by ACE and its receptor mediated functions through regulation of both the AT1 and AT2 receptors.

Keywords: Pleiotrophin; Angiotensin converting enzyme; Angiotensin II; Aorta

Pleiotrophin (PTN the protein, *Ptn* the gene), also known as Hb-GAM, is a secretory heparin-binding cytokine [1–3] with diverse roles in both development and in adulthood ([4–7], summarized in [8]). The human, bovine, mouse, and rat PTN proteins are very highly conserved and share over 50% identity in amino acid sequence with midkine (MK) [2]. Together, *Ptn* and *Mk* are the only members of the *Ptn* growth/differentiation gene family. The *Ptn* gene is strictly regulated in temporal sequence and specificity of cell type expression during development but, in adults, *Ptn* gene expression levels are stable but limited to only few cell types [7,9]; however, *Ptn* gene expression is markedly upregulated after injury in adults, in cells such as inflammatory macrophages, and endothelial cells and microglia [10–12]. The *Ptn* gene is also a protooncogene [13–15] and, an activated endogenous *Ptn* gene has been found in many cell lines derived from aggressive human malignancies [14,16–20]; importantly, in those cell lines studied, expression of the endogenous *Ptn* gene is constitutive

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and PTN signaling has been demonstrated to be a major contributor to the highly malignant phenotype of these cells [14,19]. These different contexts in which the endogenous *Ptn* gene is expressed suggest that many diverse genetic pathways are regulated through an activated *Ptn* gene and the need to better define the genetic programs initiated by PTN signaling.

The structure of PTN supports the possibility that PTN signals diverse phenotypes in different contexts. The mature PTN protein of 136 amino acids has lysine rich domains at the extremes of each of the N- and C-termini [2] and separate, heparin-binding thrombospondin type I repeat-like domains internally in each of the N- and C-terminal domains each of which is coupled centrally through a short linker region [21]. These separate domains of PTN when expressed alone in cells with the endogenous signal peptide have been found to signal independently discrete but different phenotypes of each other and thus, the separate PTN domains are likely to be recognized by independent receptor-like molecules and to initiate independent signaling pathways. The separate signaling domains within the PTN molecule and the separate signaling pathways they trigger establish an additional basis for the diversity of PTN functional responses cited below and thus enlarge the pool of genes whose regulation depends upon an activated *Ptn* gene.

Among the different functional activities attributed to PTN, it has been found to stimulate proliferation of different cell lines in culture, including epithelial cells, endothelial cells, and fibroblasts [1,2,22–24], and to stimulate lineage-specific differentiation of glial progenitor cells [25] and neurite outgrowth from neonatal neuronal cells in primary culture [1–3]. Pleiotrophin also stimulates angiogenesis in vivo and in vitro by different criteria [10,23,24,26–28]. When injected directly into ischemic rat myocardium, PTN signals functionally new capillary and arteriolar formation [29]. When an ectopic *Ptn* gene is expressed in premalignant cells and these cells are injected into flanks of nude mice, the premalignant cells with the ectopic *Ptn* gene expression establish subcutaneous tumors with the striking new phenotypes of rapid tumor growth and extensive tumor angiogenesis [13,23].

Pleiotrophin signals at least in part through its ability to interact with and enforce dimerization of the receptor protein tyrosine phosphatase (RPTP) β/ζ [8,30] and, as a consequence of a presumed PTN-dependent conformational change that blocks access of substrate phosphorylated proteins to the active site of RPTP β/ζ, to ablate the intrinsic activity of RPTP β/ζ. Loss of the intrinsic tyrosine phosphatase activity of RPTP β/ζ increases the steady state levels of tyrosine phosphorylation of those substrates of RPTP β/ζ, whose steady state levels of tyrosine phosphorylation are maintained by the endogenous tyrosine phosphatase activity of RPTP β/ζ and the tyrosine kinase activity of a constitutively active but unknown protein tyrosine kinase(s). Substrates of RPTP β/ζ identified include β-catenin [30] and adducin 2β [31]. The consequence of increased tyrosine phosphorylation of β-catenin is disruption of cytoskeletal structure and loss of cell–cell adhesion [32,33]. β-Catenin also is an important transcriptional transactivator, suggesting PTN signaling through RPTP β/ζ and increased tyrosine phosphorylation of β-catenin may lead to nuclear translocation of β-catenin and transcriptional activation of the new genetic pathways and thus to signal additional phenotypic diversities.

The goal in this and a previously published manuscript [34] was to further identify the pathways leading to the complexity of phenotypes that are the consequences of PTN signaling. We have analyzed and compared the transcriptional profiles of 14,400 genes in aortae of *Ptn* −/− and wild type (WT, *Ptn* +/+) mice, and used these data to define the different roles that PTN may have in specification of the phenotype of the aortae of WT, *Ptn* +/+ mice. The initial analysis cited above uncovered the unique finding that PTN is a critical regulator of the enzymes of catecholamine biosynthesis [34]. In these experiments, we demonstrate that the renin–angiotensin system is regulated by PTN, extending significantly the range of different phenotypes which are dependent on *Ptn* gene activation during development and in adults at sites of upregulation of the *Ptn* gene.

Materials and methods

Genotyping of Ptn −/− mice. The *Ptn* −/− mice were generated as previously described [34,35]. The animals used in this study were male *Ptn* −/− and WT (*Ptn* +/+) mice at 8 weeks of age. The genotypes of the *Ptn* −/− mice were confirmed prior to sacrifice with the polymerase chain reaction using as primers 5′-GAT TGA ACA AGA TGG ATT GC-3′ forward and 5′-CAT TTA GGC AAA CAG GAA GGA CG-3′ reverse to generate from genomic DNA extracted from tails of *Ptn* −/− and WT (*Ptn* +/+) mice a cDNA of ~0.7 kb.

Tissue acquisition, RNA extraction, and gene chip analysis. After being anesthetized with halothane, *Ptn* −/− and WT (*Ptn* +/+) mice were sacrificed. The aortae were rapidly dissected from three animals per strain, frozen in dry ice, and stored at −80 °C before RNA isolation. Frozen tissues were homogenized in 1 ml of the TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50–100 mg tissue and total RNA was extracted following the manufacturer's protocol. The concentrations of RNA in each sample were measured by A_{260} and the integrity of RNA was confirmed in 1.25% agarose gels after electrophoresis. RNA samples were treated with a preparation of DNAses (Ambion, Austin, TX) following manufacturer's protocol. Pooled RNA from three samples of each strain was used for microarray analysis.

Affymetrix mouse 14,400 oligonucleotide probe Genome MOE 430A Gene Chips were used according to the standard protocols supplied by the manufacturer as described [34]. Different "housekeeping" genes, including actin, GAPDH, and hexokinase, and other control sequences were included as reference and quality control indicators.

cDNA synthesis and SYBR green RT-PCR analysis. Complementary DNAs were synthesized from the pool of three samples of total

RNA of aortae from WT (*Ptn* +/+) and *Ptn* −/− mice using a cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The SYBR green RT-PCR method (Bio-Rad, Hercules, CA, USA) was used to confirm results obtained in the gene chip studies using the following primer sets (forward and reverse): angiotensinogen (5′-ATCACCAACTTCGTG GGCTTC-3′, 5′-TCCAAGGTAGAAAGAGACCAGGG-3′); renin (5′-CTTGGCTGAACCAGATGGACAG-3′, 5′-GCATTTTCTTGA GTGGGATTCG-3′); ACE (5′-TGAGAAAAGCACGGAGGTAT CC-3′, 5′-AGAGTTTTGAAAGTTGCTCACATCA-3′) [36]; AT1 (5′-CCATTGTCCACCCGATGAAG-3′, 5′-TGCAGGTGACTTTGGC CAC-3′) [36]; AT2 (5′-CAGCAGCCGTCCTTTTGATAA-3′, 5′-TTA TCTGATGGTTTGTGTGAGCAA-3′) [36]; and GAPDH (5′-CCTG CACCACCAACTGCTTA-3′, 5′-TCATGAGCCCTTCCACAATG-3′) [37]. The relative expression of each gene was normalized against GAPDH, the reference standard, as described by the manufacturer's user bulletin # 2 of ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Other reference standards were tested and found to be less stable in the experiments testing WT (*Ptn* +/+) and *Ptn* −/− mice.

Results

Comparison of expression levels of key genes in the renin–angiotensin system in aortae of WT (Ptn +/+) and Ptn −/− mice

Analysis of the Affymetrix chip microarray from WT (*Ptn* +/+) mice and mice genetically deficient in the *Ptn* gene (*Ptn* −/−) identified highly significant differences in expression levels of genes encoding key proteins in the renin–angiotensin system (Fig. 1). The transcripts encoding the angiotensin converting enzyme were decreased ~3-fold in *Ptn* −/− mice relative to *Ptn* +/+ mice and the transcripts encoding angiotensin II type 1 (AT1) receptor were ~4-fold increased in *Ptn* −/− mice relative to *Ptn* +/+ mice. Transcripts encoding angiotensinogen, renin, and the angiotensin II type 2 receptor (AT2) were not detected in the microarray chip analysis of either *Ptn* −/− mice or *Ptn* +/+ mice (Table 1).

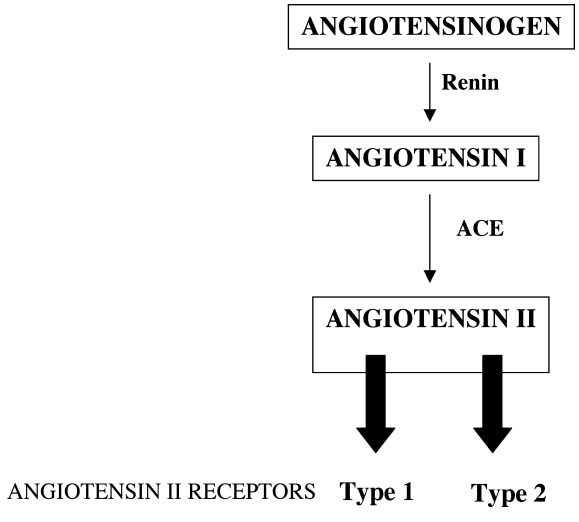


Fig. 1. Renin–angiotensin pathway.

Table 1
Upregulation of expression levels of genes of the renin–angiotensin pathway in aortae of *Ptn* −/− vs. WT (*Ptn* +/+) mice

Gene	Aorta *Ptn* −/− vs. *Ptn* +/+
Angiotensinogen	Not detected
Renin	Not detected
ACE	3-fold⇓
AT1 receptor	3-fold⇑
AT2 receptor	Not detected

The Affymetrix MOE 430A chip was used to measure simultaneously the expression levels of 14,400 genes. Total RNA isolated from three aortae of each strain was used for this study. The values show the fold regulation as a result of the ratio of the expression intensities of each gene in *Ptn* −/− vs. *Ptn* +/+.

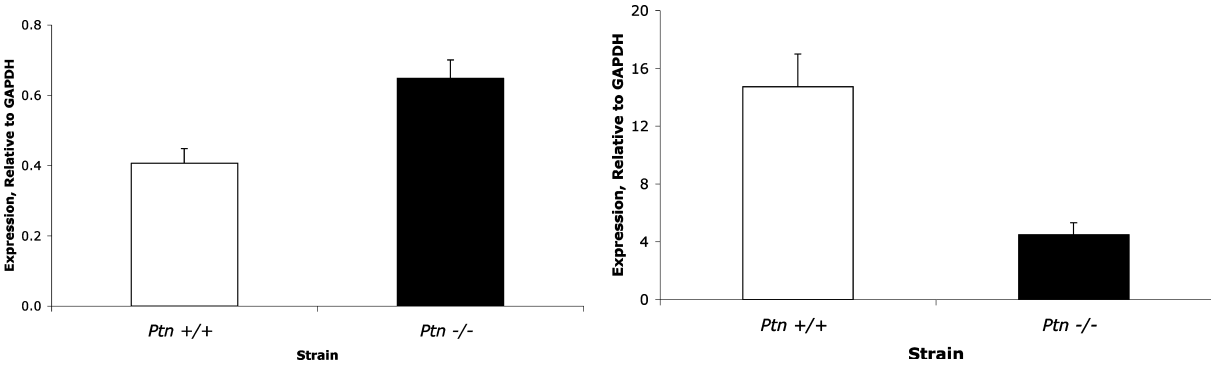
Confirmation of microarray analysis by SYBR green RT-PCR

To confirm these differences in the levels of the genes involved in the renin–angiotensin pathway identified in the microarray analysis above and to seek the expression levels of those genes beneath detection levels in the Affymetrix chip microarray analysis, triplicate determinations of expression levels of each gene in the renin–angiotensin pathway were measured using the SYBR green RT-PCR methodology described above.

In the SYBRgreen RT-PCR analysis, the transcripts of the angiotensinogen and renin were readily detected in aortae of both WT (*Ptn* +/+) and *Ptn* −/− mice. Significant differences between the levels of the transcripts of the angiotensinogen gene were not found when aortae of WT (*Ptn* +/+) and *Ptn* −/− mice were compared (data not shown). A slight increase in the mRNA levels of renin was demonstrated in aortae of *Ptn* −/− mice in comparison with aortae of WT (*Ptn* +/+) mice (Fig. 2A) but judged not to be significant. Highly significant decreases in the levels of angiotensin converting enzyme (ACE) mRNA expression were found (Fig. 2B) whereas highly significant increases in the levels of the mRNA of AT1 receptor were clearly seen in aortae of *Ptn* −/− vs. WT (*Ptn* +/+) mice (Fig. 2C). Furthermore, the transcripts of the AT2 receptor, not detected in the microarray analysis, also were readily detected and, when compared, highly significant increases in the levels of the transcripts of the AT2 receptor were demonstrated in aortae of *Ptn* −/− mice relative to aortae of WT (*Ptn* +/+) mice (Fig. 2D).

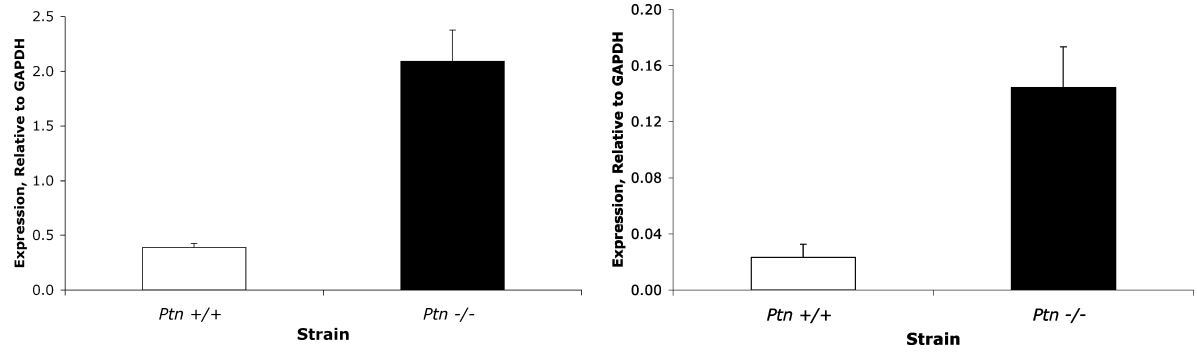
Discussion

The findings described in this paper demonstrate for first time that expression levels of key genes encoding proteins unique to the renin–angiotensin system in



(A) Renin mRNA levels in aorta.

(B) Angiotensin converting enzyme mRNA levels in aorta.



(C) Angiotensin II type 1 (AT1) receptor mRNA levels in aorta.

(D) Angiotensin II type 2 (AT2) receptor mRNA levels in aorta.

Fig. 2. Renin (A), angiotensin converting enzyme (B), angiotensin II type 1 (AT1) receptor (C), and angiotensin II type 2 (AT2) receptor (D) mRNA expression levels in aortae of *Ptn* −/− and wild type (*Ptn* +/+) mice. Messenger RNA levels were measured by SYBR green RT-PCR and are expressed in relation to the levels of GAPDH used as a housekeeping gene. Aortae from three animals of each strain were used for RNA extraction and the determination was done on one only sample of each strain coming from the pool of the three different specimens. The results are shown as average ± SE of three different determinations.

mouse aorta are regulated by expression levels of the *Ptn* gene. The results demonstrate that *Ptn* −/− mice have a highly significant decrease in the levels of ACE mRNA and, conversely, highly significant increases in levels of the AT1 and AT2 receptor mRNAs; the results thus suggest that levels of *Ptn* gene expression and presumably PTN signaling are critical regulators of the renin–angiotensin system in mouse aorta. Specifically, the data suggest that PTN may critically regulate the downstream functions of angiotensin II through regulation of expression levels of ACE, required for the enzymatic cleavage of angiotensin I to generate angiotensin II, and through the ability of PTN to regulate the levels of expression of the two angiotensin II receptors, AT1 and AT2, with which angiotensin II interacts to initiate the different functional downstream responses of angiotensin II in vivo.

The potential significance of the discovery that levels of *Ptn* gene expression regulate the levels of expression of genes unique to the renin–angiotensin pathway may be high, since the potential for levels of *Ptn* gene expression and PTN signaling to significantly impact the diverse downstream phenotypes signaled through the interactions of angiotensin II with its cognate receptors AT1 and AT2 leads directly to the possibility that levels of PTN signaling broadly impact the many angiotensin II signaled functions in vivo. Angiotensin II is an essential regulator of blood pressure [38–40] and of growth of smooth muscle cells [41–43]. The renin–angiotensin system appears to have a critical role in angiogenesis [44–46], and angiotensin II induces the expression of vascular endothelial growth factor (VEGF) under non-hypoxic conditions [47–49]. The importance of these pathways dependent upon angiotensin II signaling through the AT1 and AT2 receptors is also high. These pathways have been successfully targeted by different therapeutics to control the negative effects of the renin–angiotensin system in different diseases of the cardiovascular system, such as atherosclerosis, hypertension, and cardiac hypertrophy, and in other disorders directly targeted to vascular smooth muscle cell growth and inflammation [50–53].

Added significance to this discovery may derive from previous observations that *Ptn* gene expression is highly regulated during development in both time and cell type specificity [1–3,7,9,54], sharply upregulated in inflammatory macrophages, endothelial cells, and activated astrocytes in vivo in response to injury [10,11], and

constitutively activated in many human malignant tumors [8,14,20,55,56]. Pleiotrophin gene expression thus is normally subjected to tightly regulated cycles of high level upregulation in different physiological contexts both in development and in adults and deregulated high level *Ptn* gene expression is found in many pathological circumstances, suggesting the potentially important concept that, in the different contexts in which *Ptn* gene expression levels are significantly upregulated, the upregulated *Ptn* gene expression may significantly change angiotensin II-dependent phenotypes. Furthermore, the pathological angiotensin II-dependent phenotypes that are shown to be downstream of *Ptn* gene expression may thus be effectively targeted by existing therapeutics directed to the renin–angiotensin pathway.

The regulation of the renin–angiotensin system is complex and not yet fully understood. The expression levels of the AT1 receptor are modified in different cytokine stimulated vascular smooth muscle cells [57]. Interleukin-1α increases the angiotensin II binding to the AT1 receptor and the levels of the AT1 receptor transcripts. Interleukin-1α, TNF-α, and interferon-γ together decrease the levels of the AT1 receptor mRNA but alone, neither TNF-α nor interferon-γ affects expression levels of AT1 receptor mRNA. Stimulation of both of the AT1 and AT2 receptors by angiotensin II increases the release of IL-6, IL-8, TNF-α, and TGF-β [58–60], an effect attributed to the activation of nuclear factor (NF)-κβ [59], a nuclear factor which, when activated and translocated to the nucleus, initiates new gene expression important in the regulation of the vascular system [61].

Angiotensin II is also known to increase the levels of expression of tyrosine hydroxylase, the rate-limiting enzyme of the catecholamine biosynthesis [62–64]. This finding may be relevant to the analysis of *Ptn* −/− mice, in which we described a striking upregulation of the key enzymes of the catecholamine biosynthesis pathway in aortae of *Ptn* −/− mice when compared with WT (*Ptn* +/+) mice [34], suggesting a complex interactive mechanism in which *Ptn* may influence catecholamine biosynthesis through angiotensin II in aortae. These complexities and their possible importance in different functions of aorta emphasize the need for additional experiments to more deeply understand the mechanisms and significance of regulation of the renin–angiotensin pathway.

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Fyn is a downstream target of the pleiotrophin/receptor protein tyrosine phosphatase β/ζ-signaling pathway: Regulation of tyrosine phosphorylation of Fyn by pleiotrophin

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Abstract

Pleiotrophin (PTN the protein, *Ptn* the gene) signals downstream targets through inactivation of its receptor, the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ, disrupting the balanced activity of RPTPβ/ζ and the activity of a constitutively active tyrosine kinase. As a consequence of the inactivation of RPTPβ/ζ, PTN stimulates a sharp increase in the levels of tyrosine phosphorylation of the substrates of RPTPβ/ζ in PTN-stimulated cells. We now report that the Src family member Fyn interacts with the intracellular domain of RPTPβ/ζ in a yeast two-hybrid system. We further demonstrate that Fyn is a substrate of RPTPβ/ζ, and that tyrosine phosphorylation of Fyn is sharply increased in PTN-stimulated cells. In previous studies, we demonstrated that β-catenin and β-adducin are targets of the PTN/RPTPβ/ζ-signaling pathway and defined the mechanisms through which tyrosine phosphorylation of β-catenin and β-adducin disrupts cytoskeletal protein complexes. We conclude that Fyn is a downstream target of the PTN/RPTPβ/ζ-signaling pathway and suggest that PTN coordinately regulates tyrosine phosphorylation of β-catenin, β-adducin, and Fyn through the PTN/RPTPβ/ζ-signaling pathway and that together Fyn, β-adducin, and β-catenin may be effectors of the previously described PTN-stimulated disruption of cytoskeletal stability, increased cell plasticity, and loss of cell–cell adhesion that are characteristic of PTN-stimulated cells and a feature of many human malignant cells in which mutations have established constitutive expression of the *Ptn* gene.

Keywords: Pleiotrophin; Fyn; Receptor protein tyrosine phosphataseβ/ζ; Cytoskeletal stability

Pleiotrophin (PTN the protein, *Ptn* the gene) is a 136 amino acid highly conserved, secreted heparin binding cytokine [1–3]. Pleiotrophin shares more than 50% identity in amino acid sequence with midkine (MK the protein, *Mk* the gene); together, PTN and MK constitute the only members of this family of developmentally regulated cytokines [1,3,4]. Pleiotrophin expression levels peak during embryonic development later than those of *Mk* and PTN thus is downstream of *Mk* and likely to be significantly functionally redundant with PTN [5,42,41].

The *Ptn* gene is expressed in different cell types during development [1,3,6–10]. However, in adults, *Ptn* gene expression is constitutive and limited to only a few cell types [6]. However, the *Ptn* gene is upregulated in inflammatory macrophages and endothelial cells at sites of injury [8].

The *Ptn* gene also is a proto-oncogene. It transforms NIH 3T3 cells [11] and, in these cells, it induces a disordered cytoskeleton, increased motility, and other properties of the highly malignant phenotypes that are

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characteristic of the human malignant cells that express an activated endogenous *Ptn* gene [4,10,12,46]. Introduction of an activated *Ptn* gene into pre-malignant cells "switches" the pre-malignant cells to stages of higher malignancy [12,13,46] and, in malignant cells derived from human tumors with mutations that initiate constitutive activation of the endogenous *Ptn* gene, ablation of the endogenous PTN-signaling pathway by introduction of a dominant negative *Ptn* gene or targeted *Ptn* ribozymes effectively reverts the highly malignant phenotype of these cells to that of the pre-malignant cell, demonstrating that constitutive *Ptn* signaling alone is necessary and sufficient to initiate and sustain a highly malignant phenotype [10,12,14,15,40].

One receptor that transmits a PTN-dependent signal is the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ. Pleiotrophin signals through interaction with and the consequent inactivation of the endogenous protein tyrosine phosphatase activity of RPTPβ/ζ. As a result, the steady-state levels of tyrosine phosphorylation of substrates of RPTPβ/ζ including β-catenin [16] and β-adducin [43,44] increase due to the unbalanced persistent tyrosine kinase(s) activity acting at the same site dephosphorylated by RPTPβ/ζ. Pleiotrophin thus signals through its ability to critically regulate steady-state levels of tyrosine phosphorylation of downstream target proteins through inactivation of RPTPβ/ζ.

β-Catenin is the first downstream target of the PTN/RPTPβ/ζ-signaling pathway to be discovered [16]. It is a critical regulator of cytoskeletal stability and function; it links α-catenin and the highly conserved cytoplasmic domains of cadherins with the actin cytoskeleton [17–19]. The PTN/RPTPβ/ζ pathway stimulates increase in tyrosine phosphorylation of β-catenin, disrupts the association of β-catenin with the cytoplasmic domain of the cadherins, destabilizes adherins junctions, and decreases homophilic cell–cell adhesion [10,16,45].

β-Adducin is the second downstream target of the PTN/RPTPβ/ ζ-signaling pathway to be identified [43,44]. It belongs to a family of proteins that bind to actin–spectrin junctions [20–22] and stabilize the growing actin filaments and actin–spectrin networks [23,24]. PTN activates protein kinase C (PKC) and stimulates the PKC-catalyzed phosphorylation of serines 713, 726 in the MARCKS domain of β-adducin and stimulates the translocation of β-adducin phosphorylated in serines 713, 726 to either nuclei where it is associated with nuclear chromatin and with centrioles of dividing cells or to a membrane associated site, depending on the phase of cell growth. Since phosphorylation of serines 713, 726 in β-adducin markedly reduces the affinity of β-adducin for spectrin and actin, and uncouples actin/spectrin/β-adducin multimeric complexes needed to stabilize the cytoskeleton, the PTN-stimulated phosphorylation of serines 713, 726 in β-adducin contributes to the disruption of cytoskeletal complexes and thus is an important component of the previously demonstrated loss of cytoskeletal integrity and homophilic cell–cell adhesion in PTN-stimulated cells [43,44]. The discoveries that PTN through the PTN/RPTPβ/ζ-signaling pathway phosphorylates both β-catenin and β-adducin, and through them, initiates cytoskeletal disruption, illustrate clearly the importance of PTN in the regulation of cytoskeletal structure.

To identify other proteins that may be regulated through the PTN/RPTPβ/ζ-signaling pathway and of potential significance in cytoskeletal regulation, we screened a human fetal brain cDNA expression library with the intracellular domain of RPTPβ/ζ using a yeast two-hybrid interactive protein system, an effective and frequently used system to identify protein–protein interactions [25]. We identified the Src kinase family member Fyn to interact with the intracellular domain of RPTPβ/ζ. Because Fyn signaling is known to mediate diverse cytoskeletal functions, the relationship of Fyn with RPTPβ/ζ suggested that Fyn also may be a mediator of the PTN-stimulated cytoskeletal phenotype.

Materials and methods

Yeast two-hybrid screen. AH109 competent yeast cells, the yeast expression vectors pGBKT7 and pACT1, and the yeast cells Y187 pretransformed human fetal brain MATCHMAKER cDNA Library were obtained from BD Biosciences Clontech (La Jolla, CA). The human fetal brain library was selected because of the previously observed high-level expression of RPTPβ/ζ and PTN in early brain development [6], suggesting that proteins in the PTN-signaling pathway may be expressed in high levels in brain at that time. The full-length RPTPβ/ζ clone (Fig. 1) (GenBank Accession No. NM_002851) was a generous gift from H. Saito, Dana Farber Cancer Center, Boston, MA. The

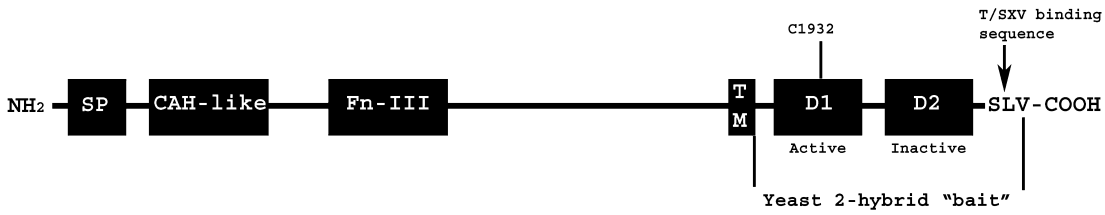

Fig. 1. Schematic representation of receptor protein tyrosine phosphatase (RPTP)β/ζ. SP, signal peptide; CAH-like, carbonic anhydrase-like domain; Fn-III, fibronectin type III domain containing chondroitin sulfate; TM, transmembrane domain; D1, active tyrosine phosphatase domain (C1932, phosphatase catalytic residue); D2, inactive tyrosine phosphatase domain; SLV, C-terminal PDZ binding sequence. Yeast two-hybrid "bait," residues 1663–2314.

cytoplasmic domain of RPTPβ/ζ (residues 1663–2314) (see Fig. 1) was amplified using polymerase chain reaction (PCR) and subcloned into the yeast expression vector pGBKT7 for transformation of AH109 competent yeast cells. AH109 cells expressing RPTPβ/ζ residues 1663–2314 were confirmed by DNA sequencing analysis and Western blots of the induced AH109 cell lysates probed with anti-RPTPβ/ζ specific antibodies (BD Transduction Laboratory, San Diego, CA). The confirmed strain was co-cultured with compatible Y187 yeast cells containing the pre-transformed human fetal brain library overnight in a shaker at 30 °C in 50 ml YPAD yeast media. The mating yeast were plated on SD medium-stringency selection plates (SD/−His/−Leu/−Trp) and surviving colonies were re-plated on high-stringency SD medium (SD/−His/−Leu/−Trp/−Ade). The colonies stained blue were tested for β-galactosidase activity with a colony filter-lift assay, the isolated library clones confirmed to be positive were purified, the cDNAs were sequenced, and the encoded proteins were identified by screening different standard databases.

To confirm the interaction of the isolated clones with the cytoplasmic domain of RPTPβ/ζ, AH109 cells were co-transformed with the vector pGBKT7-RPTPβ/ζ cytoplasmic domain and the vector pACT2 containing the brain library clone whose interaction was characterized as above. The isolated clones were confirmed for their ability to grow on the highest stringency selection plates and expression of α- and β-galactosidase.

Preparation of glutathione-S-transferase fusion proteins. The active site containing D1 domain of RPTPβ/ζ (residues 1663–2034) and an inactivated active site RPTPβ/ζ D1 domain mutant (residues 1663-2034, C1932S) were prepared by inserting the cDNA fragment encoding the human RPTPβ/ζ, amino acids 1663–2034 or 1663–2034 (C1932S) fused with GST to the bacterial expression plasmid pGEX-KG (Amersham Pharmacia, Piscataway, NJ). The GST fusion proteins have been termed GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932S), respectively. The constructs (or GST alone) were expressed in BL-21 competent cells grown in 100 ml Luria–Bertani broth overnight, grown to middle log phase, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h, and lysed with 1% Triton X-100 in PBS with 0.1% phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, and 1× Complete Protease Inhibition Cocktail (Roche Applied Sciences, Indianapolis, IN). The GST and GST fusion proteins were immobilized with 100 μl of glutathione–Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ) and washed with 1% Triton X-100 in PBS. The GST-RPTPβ/ζ D1 fusion protein activity was tested by measuring its ability to dephosphorylate β-catenin phosphorylated in tyrosine to confirm it is an active tyrosine phosphatase. GST-RPTPβ/ζ D1 (C1932S) was shown to bind to, but not to dephosphorylate, β-catenin phosphorylated in tyrosine as previously described [16].

Detection of Fyn phosphorylated in tyrosine. Fyn was immunoprecipitated from cell lysates as indicated below with 6 μg anti-Fyn antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to 50 μl of protein G–Sepharose beads and incubated overnight at 4 °C with gentle mixing. The protein G coupled anti-Fyn antibody beads incubated with the cell lysates were washed, eluted, and analyzed by Western blots probed with anti-phosphotyrosine antibodies (BD Transduction Laboratory, San Diego, CA). The blots were stripped and re-probed with anti-Fyn antibodies to ensure that the protein recognized by anti-phosphotyrosine antibodies was Fyn.

Dephosphorylation of Fyn phosphorylated in tyrosine by RPTPβ/ζ. Three 100 mm plates of HeLa cells were grown to 70% confluence in DMEM with 10% FBS, serum starved for 24 h, and stimulated with PTN 50 ng/ml (R&D Systems, Minneapolis, MN) for 60 min. The cells were lysed and samples were incubated with either GST-RPTPβ/ζ D1 for 10, 20, and 60 min or GST-RPTPβ/ζ D1 (C1932S) for 60 min in 25 mM Tris–HCl, pH 7.2, 10 mM DTT in PBS at 37 °C. After the incubation, Fyn was immunoprecipitated from the lysates with 6 μg anti-Fyn antibodies coupled to 50 μl protein G–Sepharose beads and incubated overnight at 4 °C. Protein G coupled Fyn was washed with 1% Triton X-100 in PBS, pH 7.2, eluted, and analyzed in Western blots probed with anti-phosphotyrosine antibodies (BD Transduction Laboratory, La Jolla, CA). The blots were stripped and re-probed with anti-Fyn to confirm the identity of Fyn.

Results

Yeast two-hybrid screen: identification and confirmation of Fyn as interactive with the cytoplasmic domain of RPTPβ/ζ in yeast

Eighty-eight yeast colonies grew under intermediate stringent nutrient selective conditions. Twelve of these colonies grew well under the most stringent nutrient selective conditions when the human fetal brain library was screened with the intracellular domain of RPTPβ/ζ ("bait") in the yeast two-hybrid system (Fig. 1). The cDNAs from each of the 12 clones were sequenced; one clone encoded the in-frame, full-length human Fyn protein (Fig. 2). To confirm Fyn as interactive with the intracellular domain of RPTPβ/ζ in yeast, A109 yeast cells were transformed with the plasmid encoding the RPTPβ/ζ cytoplasmic domain and the plasmid encoding the full-length Fyn. The yeast cells grew rapidly, formed large colonies under the most stringent nutrient selective conditions, and expressed high levels of the reporter gene β-galactosidase.

Fyn is a protein tyrosine kinase of ~60 kDa with many diverse functions, including functions in cell proliferation and cell adhesion, cytoskeletal structure, and cell cycle regulation [26–32]. Fyn contains two

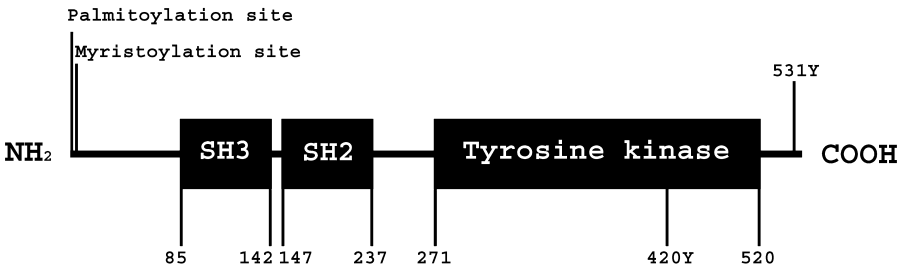

Fig. 2. Schematic representation of Fyn. Represented are the Src-homology (SH)3 domain and SH2 domain. Fyn contains two regulatory tyrosines, 420 and 521, N-terminal palmitoylation and myristoylation sites.

previously recognized tyrosine phosphorylation sites (EDNEY(420)TARQ, TEPQY(531)QPGE) (Fig. 2) [26] raising the possibilities that Fyn is a substrate of RPTPβ/ζ and a downstream target of the PTN/RPTPβ/ζ-signaling pathway.

Pleiotrophin increases the steady-state levels of tyrosine phosphorylation of Fyn

To address these possibilities, confluent non-stimulated HeLa cells and HeLa cells stimulated with 50 ng/ml PTN for 5, 15, 30, and 60 min were harvested, and cell lysates were prepared and used to immunoprecipitate Fyn. The immunoprecipitates were separated by SDS–PAGE, analyzed in Western blots probed with anti-phosphotyrosine antibodies, stripped, and re-probed with anti-Fyn antibodies to confirm the identity of the phosphorylated protein as Fyn. The results demonstrate that PTN stimulates a time-dependent increase in tyrosine phosphorylation of Fyn in PTN-stimulated HeLa cells (Fig. 3). A significant increase in tyrosine phosphorylation is seen 5 min after stimulation with PTN and increased levels of tyrosine phosphorylation are seen up to 60 min after the cells were stimulated with PTN (Fig. 3, upper panel). Fyn in cells not stimulated with PTN was found to have readily detected baseline levels of tyrosine phosphorylation.

The data thus indicate that the levels of tyrosine phosphorylation of Fyn are sharply increased in PTN-stimulated cells, and thus that Fyn is a downstream target of the PTN/RPTPβ/ζ-signaling pathway.

Fyn is a substrate of RPTPβ/ζ

PTN stimulates tyrosine phosphorylation of downstream targets through the inactivation of RPTPβ/ζ leaving unchecked the persistent activity of an unknown tyrosine kinase to phosphorylate substrates of RPTPβ/ζ, and thus to increase the levels of tyrosine phosphorylation of the substrates of RPTPβ/ζ in PTN-stimulated cells [16]. The stimulation of tyrosine phosphorylation of Fyn in PTN-stimulated cells is consistent with the hypothesis that Fyn is a substrate of RPTPβ/ζ, and thus a downstream target of the PTN/RPTPβ/ζ-signaling pathway. To support this hypothesis, lysates of control, non-stimulated cells and cells stimulated with PTN for 60 min were immunoprecipitated with anti-Fyn antibodies and the immunoprecipitates were incubated with either GST-RPTPβ/ζ D1 or with GST-RPTPβ/ζ D1 (C1932S) for different times (Fig. 4). GST-RPTPβ/ζ D1 is the D1 domain of RPTPβ/ζ which contains the active tyrosine phosphatase domain of RPTPβ/ζ fused with glutathione-*S*-transferase (GST) at its N-terminus. In GST-RPTPβ/ ζ D1 (C1932S), the RPTPβ/ζ tyrosine phosphatase active site cysteine (C1932) is inactivated. The data confirm that PTN stimulates an increase in tyrosine phosphorylation in PTN-stimulated cells (Fig. 4, lane 1 compared with lane 2). The data demonstrate that the levels of tyrosine phosphorylation of Fyn in the lysates of PTN-stimulated cells incubated with GST-RPTPβ/ζ D1 were progressively reduced with increasing times of incubation (Fig. 4, lanes 3–5), demonstrating the time-dependent dephosphorylation of Fyn by the D1 active site containing domain of RPTPβ/ζ. The levels of phosphotyrosine after 60 min of incubation with GST-RPTPβ/ζ D1 (Fig. 4, lane 5) are lower than the baseline levels of tyrosine phosphorylation of Fyn in cells not stimulated with PTN, indicating that the non-stimulated levels of tyrosine phosphorylation also are dephosphorylated by RPTPβ/ζ. In contrast, the levels of tyrosine phosphorylation of Fyn in lysates from PTN-stimulated cells incubated with the inactivated GST-RPTPβ/ζ D1 (C1932S) were not reduced (Fig. 4, lane 6).

The data establish that not only is phosphorylation of Fyn increased in PTN-stimulated cells, thus suggesting that Fyn is a downstream target of the PTN/RPTPβ/ζ-signaling pathway, but demonstrate directly that Fyn is a substrate of RPTPβ/ζ, and thus it is the PTN/RPTPβ/ζ pathway that is a regulator of tyrosine

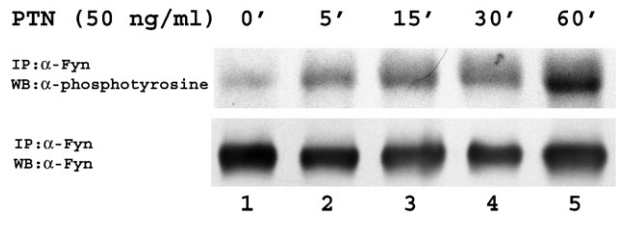

Fig. 3. Tyrosine phosphorylation of Fyn in PTN-stimulated HeLa cells. Confluent HeLa cells were stimulated with PTN (50 ng/ml) for 5, 15, 30, and 60 min. Lysates were prepared, immunoprecipitated with anti-Fyn antibodies, and analyzed in Western blots probed with anti-phosphotyrosine antibodies and re-probed with anti-Fyn antibodies. PTN stimulates a time-dependent increase in tyrosine phosphorylation of Fyn.

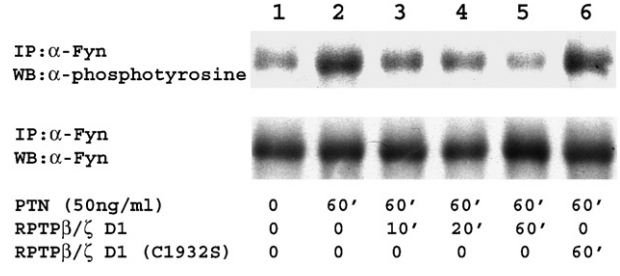

Fig. 4. Fyn is dephosphorylated by RPTPβ/ζ. Lysates from HeLa cells not stimulated and stimulated with PTN for 60 min were immunoprecipitated with anti-Fyn antibodies and incubated with the RPTPβ/ζ D1 (phosphatase active) for 10 min (lane 3), 20 min (lane 4), and 60 min (lane 5) or RPTPβ/ζ D1 (C1932S) (phosphatase inactive) domain of RPTPβ/ζ (lane 6) in Western blots probed with an anti-phosphotyrosine antibodies and re-probed with anti-Fyn antibodies.

phosphorylation of Fyn. Furthermore, because Fyn is phosphorylated in tyrosine in non-stimulated cells, the data suggest that the product of the endogenous *Ptn* gene may function to disrupt the steady-state levels of tyrosine phosphorylation of Fyn through inactivation of the endogenous RPTPβ/ζ-signaling pathway.

Discussion

In the experiments described above, a yeast two-hybrid screen was used to seek downstream protein targets of the PTN/RPTPβ/ζ-signaling pathway. One positive clone identified in the human fetal brain cDNA library screened with the intracellular domain of RPTPβ/ζ encoded the full-length human Fyn, a member of the Src family of protein tyrosine kinases. The data support strongly the conclusion that Fyn is a new member of the PTN/RPTPβ/ζ targeted signaling pathway. The data suggest that the steady-state levels of tyrosine phosphorylation may also be regulated through the endogenous PTN/RPTPβ/ζ-signaling pathway through an autocrine mechanism when cells are not stimulated by exogenous PTN but they are sharply upregulated when cells are exposed to exogenous PTN.

This study thus has uncovered a novel PTN-signaling pathway that targets Fyn and regulates its steady-state levels of tyrosine phosphorylation. Fyn has been described previously to be a substrate of RPTPα and RPTPε [32,33], suggesting that its regulation is complex and likely is the coordinated result of different transmembrane tyrosine phosphatases and multiple protein tyrosine kinases.

The increased levels of tyrosine phosphorylation of Fyn in PTN-stimulated cells identified in this study coupled with the functions of β-catenin and β-adducin that destabilize the cytoskeleton in PTN-stimulated cells [43–45] support strongly that the known critical roles of Fyn in cell adhesion, cell migration, cell proliferation, and cytoskeletal stability [34–37] may also be regulated by PTN. They suggest that tyrosine phosphorylation of Fyn may contribute in parallel with β-catenin and β-adducin to the previously demonstrated cytoskeletal destabilization and loss of calcium-dependent homophilic cell–cell adhesion that is consequent to PTN signaling previously described in PTN-stimulated cells and that also is characteristic of the different highly malignant cells that express the endogenous *Ptn* gene. Since PTN activates PKC [43,44] and PKC activates Fyn [38,39], it is also possible that PTN may also increase serine phosphorylation levels of Fyn through regulation of PKC, increasing the levels of tyrosine kinase activity of Fyn [38], and thus Fyn, in turn, may be one kinase that phosphorylates β-catenin in PTN-stimulated cells.

The data and previously reported [16,43,44] data identifying the downstream targets of the PTN/RPTPβ/ζ-signaling pathway support the hypothesis that PTN regulates the steady-state levels of tyrosine phosphorylation of β-catenin, β-adducin, and Fyn with a high degree of coordination and that the PTN/RPTPβ/ζ-signaling pathway is likely to be a central regulator of cytoskeleton structure and function in the development and some highly malignant cells that express the endogenous *Ptn* gene [1,3,4,6–10,12,46].

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Pleiotrophin induces formation of functional neovasculature in vivo

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Abstract

Pleiotrophin (PTN) is a heparin-binding growth/differentiation inducing cytokine that shares 50% amino acid sequence identity and striking domain homology with Midkine (MK), the only other member of the *Ptn*/*Mk* developmental gene family. The *Ptn* gene is expressed in sites of early vascular development in embryos and in healing wounds and its constitutive expression in many human tumors is associated with an angiogenic phenotype, suggesting that PTN has an important role in angiogenesis during development and in wound repair and advanced malignancies. To directly test whether PTN is angiogenic in vivo, we injected a plasmid to express PTN into ischemic myocardium in rats. Pleiotrophin stimulated statistically significant increases in both normal appearing new capillaries and arterioles each of which had readily detectable levels of the arteriole marker, smooth muscle cell α-actin. Furthermore, the newly formed blood vessels were shown to interconnect with the existent coronary vascular system. The results of these studies demonstrate directly that PTN is an effective angiogenic agent in vivo able to initiate new vessel formation that is both normal in appearance and function. The data suggest that PTN signals the more "complete" new blood vessel formation through its ability to stimulate different functions in different cell types not limited to the endothelial cell.

Keywords: Myocardial infarction; Angiogenesis; Perfusion; Gene therapy

Angiogenesis is the process by which new blood vessels sprout from pre-existent vasculature [1–3]; angiogenesis is highly active during embryonic development, but in adults, angiogenesis is effectively quiescent [4], unless rapidly activated by different physiological stimuli, such as seen in wound healing and female reproductive cycles or in the "pathological angiogenesis" that is frequently a feature of various diseases, notably highly advanced malignant tumors [1,5–7] and diabetic retinopathy [8,9]. Many signaling molecules, such as members of the vascular endothelial growth factor (VEGF) and the angiopoietin (Ang) families and the acidic and basic fibroblast growth factors (FGFs), have been found to have roles in the stimulation of the complex process of angiogenesis. The endothelial cell alone is the principle target of these factors. Recently, these factors have been directly tested in vivo to see if they can stimulate angiogenesis in different models but, in each case, the newly formed vessels are incomplete and/or have raised a spectrum of significant adverse effects [10–12]. In these experiments, we have tested directly whether pleiotrophin (PTN the protein, *Ptn* the gene) is an angiogenesis factor and whether PTN stimulates a "complete" neovascular response.

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Pleiotrophin is a secreted, highly conserved heparin-binding cytokine [13,14]. Pleiotrophin, also known as Hb-GAM [15], is more than 50% identical in amino acid sequence and shares striking structural and functional similarities with midkine (MK the protein, *Mk* the gene), the only other member of the *Ptn*/*Mk* developmental gene family [13–15]. Pleiotrophin gene expression is activated at sites of angiogenesis during development [16] and upregulated in endothelial and inflammatory cells at sites of new vessel formation in ischemic rat brain [17], suggesting its role in new blood vessel formation. Pleiotrophin stimulates proliferation of cultured fibroblasts [13,14,18], endothelial cells [17,19–21], epithelial cells [16,20,22–24], and furthermore, stimulates different progenitor cells in primary culture to enter lineage-specific differentiation pathways [13,14,17,25]. The *Ptn* gene also is a proto-oncogene [16,26]. The endogenous *Ptn* gene is activated constitutively with high frequency by mutations in different highly malignant human neoplasms, neoplasms usually associated with a high degree of tumor angiogenesis [27–35]. Furthermore, when the endogenous PTN signaling pathway in these malignant cells is interrupted by introduction of a dominant negative *Ptn* gene or targeted ribozymes, the phenotype of these cells reverts to that of the pre-malignant cell and the numbers of CD31 positive new blood vessels is markedly reduced when these cells are tested in the flanks of nude mice [16,22,34,36,37]. Pleiotrophin also initiates a striking increase in the appearance of CD31 positive new blood vessels when pre-malignant SW-13 (adrenal carcinoma) cells expressing an exogenous *Ptn* gene are tested in the flanks of nude mice; a structurally and functionally independent signaling domain has been identified in PTN that recapitulates fully the striking angiogenic phenotype of the full-length PTN protein when expressed in pre-malignant cells [16,18,22,24]. Recently, both PTN and MK were discovered to be required for the normal differentiation of both the catecholamine and angiotensin pathways during development and for the synthesis of specific cohorts of procollagen genes and elastin in aorta [38,39]; PTN thus signals multiple pathways that contribute in different ways to the support of a more "complete" vascular development that are signaled by different factors that directly target the endothelial cell.

The properties of PTN described above led us to test the potential of PTN to initiate a more complete angiogenic response in vivo [13,14]. The model that has been selected is ischemic tissues [40–42], since tissue remodeling and growth is limited in this context. In these studies, we attempted to facilitate reconstitution of new blood vessels in continuity with pre-existent vasculature in ischemic myocardium. We utilized a plasmid containing the *Ptn* gene to express and deliver PTN to ischemic myocardium. We now demonstrate that exogenous PTN is an effective angiogenic factor to stimulate functional or "complete" neovasculature, including stimulation of both new capillaries and arterioles.

Materials and methods

PTN expression constructs. The 580 base pair human *Ptn* open reading frame (ORF) was generated by RT-PCR at cDNA isolated from a human adenocarcinoma cell line (SW13) known to express *Ptn*, and subcloned into the *Hin*dIII and *Xba*I sites of pRc/CMV2 (Invitrogen) to generate pRC/CMV2-*Ptn*. The CMV promoter/enhancer and the *Ptn* open reading frame were shuttled from pRC/CMV2-*Ptn* to pIRES (BD Biosciences Clontech) to generate pCMV-*Ptn*-IRES. The β-gal ORF from CMVβ (BD Biosciences Clontech) was shuttled into pCMV-*Ptn*-IRES to generate pCMV-*Ptn*-IRES-β-gal-neo used to inject into ischemic myocardium. The control plasmid for injections was pCMV-β-gal plasmid without *Ptn* (Invitrogen).

Induction of myocardial ischemia and plasmid injections. A previously described ischemia–reperfusion model was used in this study [43,44]. Female Sprague–Dawley rats (225–250 g) were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and a single stitch of 7-0 Ticron suture was placed under the left coronary artery. The suture was tightened to occlude the artery for 17 min and then removed to allow for reperfusion. Previous experience indicated that this technique results in an acute myocardial infarct size of ~30% of the left ventricular [45–47]. Ten minutes after occlusion, 250 μg of pCMV-*Ptn*-IRES-β-gal-neo plasmid in 50 μl saline was injected into the ischemic left ventricle (LV) of 19 rats through a 30-gauge needle. Two hundred micrograms of pCMV-β-gal plasmid in 50 μl saline was injected into the ischemic left ventricle of six rats in the control study. The chest was then closed and the animals were allowed to recover.

Preparation of sections of control and treated ischemic myocardium. To confirm the injected plasmids effectively expressed pCMV-*Ptn*-IRES-β-gal-neo, five rats injected with ICMV-*Ptn*-IRES-β-gal-neo neo were euthanized with a pentobarbital (200 mg/kg) 5 days after injection. The hearts were rapidly excised, fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura), and sectioned into 10 μm slices. Five slides, evenly distributed throughout the infarct area and containing surrounding non-infarction tissues, were then fixed with 4% formaldehyde and incubated with 40 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma) diluted 1:40 in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4(CN)_6 \cdot 3H_2O$, and 2 mM $MgCl_2$ in PBS) at room temperature overnight. The sections stained blue, indicating that the *Ptn* plasmid effectively expressed β-galactosidase activity in vivo. An additional six *Ptn* plasmid-treated rats and six control (β-gal plasmid treated) rats were euthanized 5 weeks after infarction, a time at which point remodeling in rat is effectively complete [48]. Two additional animals injected with *Ptn* plasmid were sacrificed after 3 months and processed as above. In both the 5-week and 3-month rats, the hearts were rapidly excised, fresh frozen in Tissue Tek O.C.T. freezing medium, sectioned into 10 μm slices, and stained with H&E. Five slides, equally distributed through the infarct area, were also taken from each heart and stained with an anti-smooth muscle actin antibody (1:75 dilution; Dako), and incubated with a Cy-3 conjugated anti-mouse secondary antibody (1:100 dilution; Sigma) to label arterioles [49]. The arterioles were quantified with the following criteria: (1) positive for smooth muscle labeling, (2) within the infarct scar, (3) with a visible lumen, and (4) with a diameter between 10 and 100 μm. The scar area was measured using SPOT 3.5.1 software (Diagnostic Instruments) and arteriole densities were calculated. Five additional slides were taken from each heart and stained for capillaries. Capillaries were labeled with a biotinylated Griffonia simplicifolia lectin (GS-1; Vector Labs) and visualized using the LSAB2 System (Dako) as previously described [50]. The capillaries in five high magnification fields within the infarct area of each slide were counted and vessel density was calculated and averaged across each group.

Microbead perfusion. To determine whether injection of the *Ptn* expressing plasmid stimulated the synthesis of new functional vessels, an additional six PTN-treated rats were anesthetized with an intraperitoneal injection of 200 ml of 50 mg/ml sodium pentobarbital 5 weeks after injection and perfused with fluorescent microbeads according to a previously described procedure [51]. Briefly, the rats were injected with 700 ml of 50 mg/ml nitroglycerin to ensure vasodilation, and, after 10 min, the chest was opened and the abdominal aorta was cannulated with P-50 tubing. The left atrial appendage was cut for drainage and 9 ml of saline was then perfused retrograde through the heart for approximately 1 min and 6 ml at a suspension of 0.2 mm fluorescent carboxylate-modified polystyrene beads (FluoSpheres, Molecular Probes) diluted 1:6 with PBS were perfused through the heart. The hearts were immediately harvested, rinsed with PBS, and fresh frozen in O.C.T. freezing medium. Sections from heart in 10 μm slices were prepared and examined under a Nikon TE 300 fluorescent microscope.

Results

Injection of Ptn plasmid effectively transfects cardiomyocytes

To verify in vivo the successful transfection and functional expression of pCMV-*Ptn*-IRES-β-gal-neo following its injection into ischemic myocardium, tissue sections were examined for β-gal activity following staining with X-gal solution 5 days after injection. In vivo activity was confirmed in cells exhibiting blue staining. Up to six blue-stained cells were found in areas injected with *Ptn* plasmid in each section. While only a few cells within the infarct area were transfected, the majority of cells expressing β-gal and thus the *Ptn* gene were cardiomyocytes bordering the infarct area (Fig. 1).

PTN stimulates neovasculature formation in ischemic myocardium

To determine if PTN stimulates new blood vessel formation in ischemic myocardium, pCMV-*Ptn*-IRES-β-gal-neo was injected directly into infarcted rat myocardium. At 5 weeks following injection of pCMV-*Ptn*-IRES-β-gal-neo, the density of capillaries and arterioles was determined and compared with identically treated sections from ischemic myocardium that was injected with a control plasmid (pCMV-β-gal). The capillary density in the injured myocardium injected with pCMV-*Ptn*-IRES-β-gal-neo was 1287 ± 148 capillaries per mm^2 compared with a capillary density of 970 ± 195 capillaries per mm^2 in rat myocardium injected with (control) pCMV-β-gal ($P = 0.02$, Fig. 2), an ~46% and statistically significant increase in the capillary density in the treated myocardium. The capillary density in normal, non-ischemic myocardium was 1665 ± 367 capillaries per mm^2 and thus, the treated myocardium achieved nearly 75% the density of capillaries of the non-ischemic myocardium.

The density of arterioles in pCMV-*Ptn*-IRES-β-gal-neo-treated ischemic myocardium was 10 ± 2 arterioles per mm^2, compared to 5 ± 1 arterioles per mm^2 in pCMV-β-gal-treated tissues ($P = 0.002$, Figs. 3 and 4), a two-fold and statistically highly significant increase in arteriolar vessels in the treated versus untreated ischemic myocardium. To confirm the morphological identification of arterioles as shown in Fig. 3, sections were probed with anti-α-smooth muscle cell actin. Readily

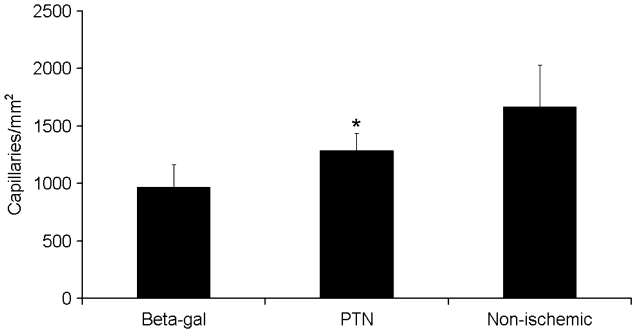

Fig. 2. Infarct capillary density. Injection of *Ptn* plasmid increased capillary density compared to injection of β-gal plasmid. $*P < 0.05$ compared to β-gal control injection, *t* test. Capillary density of normal, non-ischemic myocardium is provided as a reference.

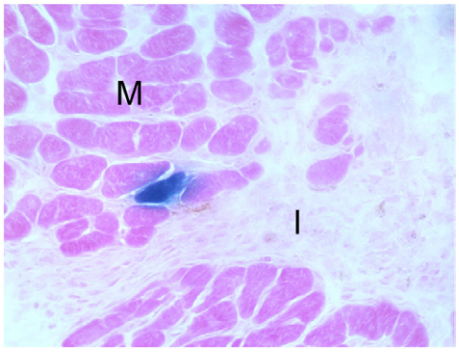

Fig. 1. In vivo transfection with the *Ptn* plasmid. Section is from myocardium 5 days following injection of the *Ptn* plasmid in saline. (M) Normal myocardium. (I) Infarcted myocardium. Note β-gal staining in a cardiomyocyte bordering the infarct area, confirming in vivo transfection with *Ptn* plasmid.

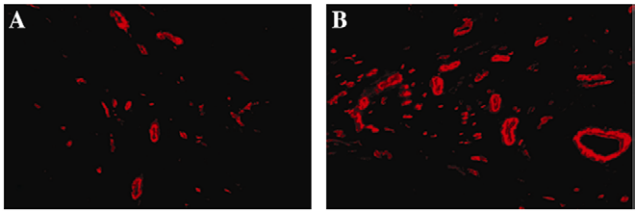

Fig. 3. *Ptn* plasmid induced arteriogenesis. Anti-α-smooth muscle actin stained arterioles 5 weeks after plasmid injection into ischemic myocardium. Arterioles in ischemic myocardium following injection of (A) β-gal plasmid and (B) *Ptn* plasmid. Note the increase in arteriole density following *Ptn* plasmid injection.

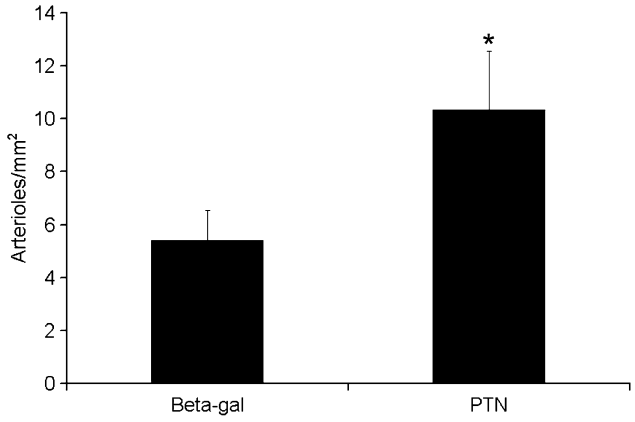

Fig. 4. Infarct arteriole density. Injection of *Ptn* plasmid increased arteriole density compared to injection of β-gal plasmid. $*P < 0.05$ compared to β-gal control injection, *t* test.

detectable levels of this marker of arterioles were found in the arteriolar structures identified by morphological criteria.

To determine if the significant increase in new capillaries and arterioles measured at 5 weeks persisted for longer periods of time, an additional two rats were injected with pCMV-*Ptn*-IRES-β-gal-neo and sacrificed after 3 months. The arteriolar (10 ± 2 arterioles per mm^2) and capillary densities (1289 ± 209 capillaries per mm^2) in sections from the treated areas of ischemic myocardium at 3 months were effectively identical to those found 5 weeks after injection. Sections stained with H&E from each animal throughout the myocardium were examined carefully; neither macroscopic nor histological evidence of vascular tumor (angioma) formation or other abnormalities were found.

Pleiotrophin-stimulated neovasculature is functionally connected to existent coronary circulation

To determine if vessels in the PTN-treated ischemic myocardium are functionally connected with the pre-existent coronary circulation, fluorescent microbeads were injected into the coronary vessels by retrograde infusion through the aorta and sections of PTN-treated ischemic myocardium were examined. The injected microbeads were present both in new vessels within the PTN-treated ischemic rat myocardium and in the vessels from the adjacent normal myocardial tissue, demonstrating that the newly formed vessels stimulated by PTN injection establish functional connections with existing coronary vessels (Fig. 5).

Discussion

The data presented in this manuscript demonstrate that the pCMV-*Ptn*-IRES-β-gal-neo plasmid injected directly into rat myocardium 5 days after ischemia–reperfusion injury stimulates significant new capillary formation by ~50% and new arteriolar formation by ~100% in the injured myocardium 5 weeks and 3 months after injury. The arterioles were confirmed as arterioles since each of them stained positively when tested with the marker anti-α-smooth muscle cell actin and, the new vessels formed in PTN-stimulated areas of ischemic myocardium were found to functionally interconnect with pre-existent coronary vasculature. Since only a few of the cardiac myocytes were found to express pCMV-*Ptn*-IRES-β-gal-neo, it remains likely that a more densely and more widely distributed injection pattern of the expression plasmid will enhance the levels of new capillaries and arterioles in the injected ischemic myocardium.

A number of previous studies have reported intramural hemangiomas and poor quality of new vessels when other angiogenic factors have been directed into ischemic myocardium [10–12]. As example, without the addition of other growth factors, VEGF is incapable of producing larger, more mature vessels such as arterioles [2]. As a consequence, without the development of coronary arteries and arterioles, the newly formed capillary beds will not be perfused. Furthermore, the capillary bed formed as a result of VEGF injection is irregular and not connected to the coronary vasculature. High doses of VEGF also produced angiomatous structures [11,12]. VEGF mRNA expression levels increase within 30 min after onset of ischemia [52–54]. Furthermore, bFGF is upregulated rapidly after ischemia as well [15], suggesting the likelihood that these previously studied angiogenic factors are involved in an acute phase in-

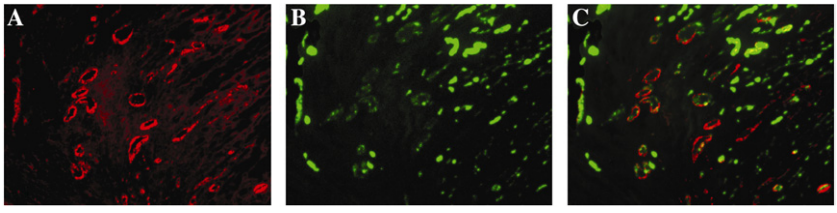

Fig. 5. Microbead perfusion. (A) Anti-smooth muscle actin labeled arterioles within PTN-treated ischemic myocardium. (B) Microbead perfusion in the same section. Microbeads are shown here in green. (C) Overlay of (A) and (B) demonstrates new vessels formed by injection of PTN are functionally connected to the coronary vasculature.

jury responses, and that they signal more limited targets primarily focused to endothelial cells, and thus they may fail to address the full needs of more complete vessels [1–3]. Following ischemia in rat myocardium, *Ptn* gene expression is not upregulated until day 3 [44,55] and, as noted above PTN signals many diverse phenotypes and it functions as a differentiation factor. It is suggested that PTN does not signal an acute phase injury response but may signal differentiation responses [22] in the context of injured myocardium, and that the more diverse downstream pathways signaled by PTN may lead to both normal capillaries and arterioles that interconnect with pre-existent coronary vessels in PTN-treated myocardium. Thus, it is speculated that PTN triggers the different signaling cascades needed for differentiation in ischemic myocardium with the scope to direct and coordinate the formation of more complete new vessels. In this context, PTN has been recently found to be required for both collagen and elastin synthesis (Herradon et al., in preparation; Ezquerra et al., in preparation), needed components of the arteriolar wall, and PTN is a critical regulator of the catecholamine biosynthesis and the renin–angiotensin pathways in mouse aorta [38,39]. The renin–angiotensin system has a critical role in angiogenesis [56,57] and, inhibitors of the angiotensin converting enzyme have been successful in suppressing angiogenesis and tumor growth [57,58], providing another pathway responsive to PTN signaling that may be important in the "complete" angiogenic response to PTN in ischemic myocardium.

Since other factors have had detrimental side effects in pre-clinical studies and mixed results in initial clinical trials [59–66], and because PTN stimulates sustained new vasculature which is interconnected with the pre-existent coronary vessels, we suggest that PTN may be a potential therapeutic angiogenic agent for use in humans.

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Midkine, a newly discovered regulator of the renin–angiotensin pathway in mouse aorta: Significance of the pleiotrophin/midkine developmental gene family in angiotensin II signaling

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Abstract

We previously demonstrated that pleiotrophin (PTN the protein, *Ptn* the gene) highly regulates the levels of expression of the genes encoding the proteins of the renin–angiotensin pathway in mouse aorta. We now demonstrate that the levels of expression of these same genes are significantly regulated in mouse aorta by the PTN family member midkine (MK the protein, *Mk* the gene); a 3-fold increase in expression of renin, an 82-fold increase in angiotensinogen, a 6-fold decrease in the angiotensin converting enzyme, and a 6.5-fold increase in the angiotensin II type 1 and a 9-fold increase in the angiotensin II type 2 receptor mRNAs were found in *Mk*−/− mouse aorta in comparison with the wild type (WT, +/+). The results in *Mk*−/− mice are remarkably similar to those previously reported in *Ptn*−/− mouse aorta, with the single exception of that the levels of the angiotensinogen gene expression in *Ptn*−/− mice are equal to those in WT+/+ mouse aorta, and thus, in contrast to *Mk* gene expression unaffected by levels of *Ptn* gene expression. The data indicate that MK and PTN share striking but not complete functional redundancy. These data support potentially high levels importance of MK and the MK/PTN developmental gene family in downstream signals initiated by angiotensin II either in development or in the many pathological conditions in which MK expression levels are increased, such as atherosclerosis and many human neoplasms that acquire constitutive endogenous *Mk* gene expression by mutation during tumor progression and potentially provide a target through the renin–angiotensin pathway to treat advanced malignancies.

Keywords: Pleiotrophin; Midkine; Renin–angiotensin; Development; Cancer

Midkine (MK the protein, *Mk* the gene) and pleiotrophin (PTN the protein, *Ptn* the gene) comprise the PTN/MK developmental gene family [1–3]. Both *Mk* and *Ptn* genes are strictly regulated in temporal sequence and specificity of cell type expression during development but, in adults, both *Mk* and *Ptn* gene expression levels are stable and limited to only few cell types [4–6]. The *Mk* gene is a retinoic acid inducible gene and *Mk* gene expression levels correlate with early stages of retinoic acid-induced differentiation in the mid-gestation period of mouse embryogenesis [7–10], whereas, the *Ptn* gene is a platelet-derived growth factor (PDGF) inducible gene and its levels of expression peak 1–2 days after those of PDGF in late embryonic development and in the immediate postnatal period, at the same time that peaks of growth and differentiation in embryos are found [2,3]. The *Mk* gene is thus expressed earlier in

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development than is the *Ptn* gene; the sites of *Mk* gene expression overlap partially but not completely with those of *Ptn* [11,12]. Both *Mk* and *Ptn* genes are upregulated in different sites in response to injury and in different pathological conditions characterized by inflammation and neoplastic disease.

Pleiotrophin and MK also signal strikingly similar functional responses in vitro, presumably the consequence of the over 50% identity of amino acid sequence and striking similarity of domain structures they share; as examples, both PTN and MK stimulate proliferation of different cell types in culture, including fibroblasts, endothelial cells, and epithelial cells [1,2,13–18]. They also stimulate neurite outgrowth and other neuronal differentiation responses in neonatal neuronal cells in primary culture [1,3,19] and lineage specific differentiation of oligodendrocyte progenitor cells in primary cultures and in vivo (Yeh et al., submitted). Pleiotrophin, the only other member of this family of the PTN/MK developmental gene family, critically regulates the catecholamine [20], and the renin–angiotensin pathways [21] in mouse aorta, pathways of fundamental importance in regulating cardiovascular homeostasis. Marked increased *Ptn* gene expression are found in heart and aorta of *Mk*−/− mice, suggesting the importance of MK in heart and that upregulated expression of *Ptn* is compensatory for absent expression of *Mk* and thus PTN-signaling is functionally redundant in these organs (Herradon et al., submitted). Genetically deficient *Mk* mice have a significantly reduced neointima formation in restenosis injury models that is reversible by systemic MK administration [22] and antisense *Mk* oligodeoxyribonucleotides suppress neointima formation in vivo [23], supporting again that *Mk* gene expression is critical to the normal function of aorta. Pleiotrophin and MK also stimulate angiogenesis in vivo [13,15,24–26] (Zhang et al., submitted).

The *Ptn* and *Mk* genes are protooncogenes. Both *Ptn* and *Mk* are constitutively expressed in different human malignancies [27–35], including human breast cancers, neuroblastoma, glioblastoma, melanoma, ovary, and lung tumors [27,29,30,34,36–39]. Morpholino antisense oligomers directed against MK and a dominant negative PTN mutant entirely abolish the malignant phenotype of cells derived from different malignant tumors with activated endogenous *Mk* and *Ptn* genes [27,40]. Thus, the mutations that activate constitutive *Ptn* or *Mk* gene expression in the premalignant cell are fully sufficient to initiate signaling in the premalignant cells to convert these cells to stages of highly malignant growth.

The present experiments are directed to understanding the phenotypes dependent on expression levels of *Mk*, with the long range goal of disserting the striking complexity of the different phenotypes stimulated in both normal and pathological conditions by MK. We present here the unique finding that MK directly regulates the expression levels of genes encoding the key proteins of the renin–angiotensin pathway and demonstrate that whereas there is a striking overlap of the consequences of *Mk* expression and those of *Ptn* gene expression on key proteins of the renin–angiotensin pathway, a significant difference has been identified as well, suggesting a significant degree but not complete functional redundancy between MK and PTN regulated pathways.

Materials and methods

Confirmation and genotyping of Ptn−/− and Mk−/− mice. The *Mk*−/− mice were generated by methods essentially identical to those previously described [41]. The animals used in this study were male *Mk*−/− and WT(+/+) mice at 8 weeks of age. The assays were carried out in accordance with the NIH Guidelines for Care and Use of Laboratory Animals.

The genotypes of the *Mk*−/− mice were confirmed prior to sacrifice with the polymerase chain reaction using as primers 5′-ATC GGT TCC AAG TCC TCC CTC CGT C-3′ forward and 5′-CAC CTT CCT CAG TTG ACA AAG ACA AGC-3′ reverse to generate from genomic DNA extracted from tails of *Mk*−/− and WT(+/+) mice a cDNA of ~0.7 kb.

The *Ptn*−/− mice generated as previously described [20,42]. The genotypes of *Ptn*−/− mice were confirmed by polymerase chain reaction using as primers 5′-GAT TGA ACA AGA TGG ATT GC-3′ forward and 5′-CAT TTA GGC AAA CAG GAA GGA CG-3′ reverse to generate a cDNA of ~0.7 kb detected in agarose gels from genomic DNA extracted from tails of *Ptn*−/− and WT(+/+) mice.

Tissue acquisition, RNA extraction, and gene chip analyses. After being anesthetized with halothane, *Ptn*−/−, *Mk*−/−, and WT(+/+) mice were sacrificed, aortae were rapidly dissected from animals (3/strain), frozen in dry ice, and stored at −80 °C pending RNA isolation. Frozen tissues were homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA) per 50–100 mg tissue and total RNA was extracted following the manufacturer's protocol. The concentration of RNA in each sample was measured by A_{260} and RNA integrity confirmed in 1.25% agarose gels after electrophoresis. RNA samples were treated with a preparation of DNAses (Ambion, Austin, TX) following manufacturer's protocol and a pool of three RNA samples from each strain was used for microarray analysis.

Affymetrix mouse 14,400 oligonucleotide probe Genome MOE 430A Gene Chips were used according to standard protocols supplied by the manufacturer, as described previously [20,21]. Different "housekeeping" genes, including actin, GAPDH, and hexokinase, and other control sequences were included as reference and quality control indicators.

Complementary DNA synthesis and SYBR green RT-PCR analysis. Complementary DNAs were synthesized from the pool of three samples of total RNA of aortae WT(+/+) and *Mk*−/− strain mice using a cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The SYBR green RT-PCR method (Bio-Rad, Hercules, CA, USA) was used to confirm the results obtained in the gene chip studies. The following primer sets (forward and reverse) were used: angiotensinogen (5′-ATCACC AACTTCGTGGGCTTC-3′; 5′-TCCAAGGTAGAAAGAGACCAG GG-3′); renin (5′-CTTGGCTGAACCAGATGGACAG-3′; 5′-GCA TTTTCTTGAGTGGGATTCG-3′); ACE (5′-TGAGAAAAGCA CGGAGGTATCC-3′; 5′-AGAGTTTTGAAAGTTGCTCACATCA-3′); angiotensin receptor type 1 (AT1) (5′-CCATTGTCCACCCG ATGAAG-3′; 5′-TGCAGGTGACTTTGGCCAC-3′); angiotensin receptor type 2 (AT2) (5′-CAGCAGCCGTCCTTTTGATAA-3′; 5′-TTATCTGATGGTTTGTGTGAGCAA-3′); and GAPDH (5′-CCT GCACCACCAACTGCTTA-3′; 5′-TCATGAGCCCTTCCACAAT G-3′) [21]. The relative expression of each gene was normalized against GAPDH, the reference standard, as described by the manufacturer's

user bulletin # 2 of ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Other reference standards were tested and found to be less satisfactory.

Results

Midkine critically regulates the renin–angiotensin pathway in aortae of Mk−/− mice

In order to better understand how MK influences the cardiovascular system and whether it is functionally redundant with its close family member PTN during development, we compared the levels of the transcripts of the different components of the renin–angiotensin pathway (Fig. 1) in aortae of mice genetically deficient in *Mk* (*Mk*−/−) in gene chip microarrays. It was found that the transcripts of angiotensinogen were upregulated many-fold in aortae of *Mk*−/− compared to aortae of WT(+/+) mice. Furthermore, the levels of expression of renin and of the AT1 receptor were upregulated but the levels of the angiotensin converting enzyme (ACE) were decreased many fold. The AT2 mRNA was not detected in the microarray chip in either *Mk*−/− and WT(+/+) mouse strains. However, the failure to detect transcripts of the AT2 receptor proved artefactual, since SYBR green RT-PCR of the same sample in triplicate analysis (see below) established that the AT2 receptor mRNA levels were readily detectable.

Quantification and confirmation of the differences in expression levels of the genes of the renin–angiotensin pathway in aortae of Mk−/− mice vs. WT(+/+) mice with SYBR green RT-PCR

The results above indicate that levels of *Mk* gene expression have a profound influence on the renin–angiotensin pathway in mouse aorta; the influence is not limited to an isolated protein. To confirm and thus establish the significance of these findings and to better quantify the differences seen, triplicate determinations of each of the results above were performed, using the SYBR green RT-PCR methodology described above. The data confirmed the striking upregulation of the levels of angiotensinogen mRNA in aortae of *Mk*−/− mice (Fig. 2A); a ~82-fold increase was demonstrated. The data also confirmed an increase (~3-fold) in the levels of expression of renin (Fig. 2B), a highly significant decrease of ~6-fold in the levels of angiotensin converting enzyme (Fig. 2C), and a highly significant increase of ~6.5-fold in the levels of AT1 receptor in the aortae of *Mk*−/− mice relative to those in aortae of WT(+/+) mice (Fig. 2D). Using this more sensitive technique to detect levels of mRNA expression, highly significant increases in levels of expression of AT2 receptor (~9-fold) were then found in aortae of *Mk*−/− mice (Fig. 2E), thus identifying the absence of expression of the AT2 receptor mRNA in the microarray chip analysis as artefactual.

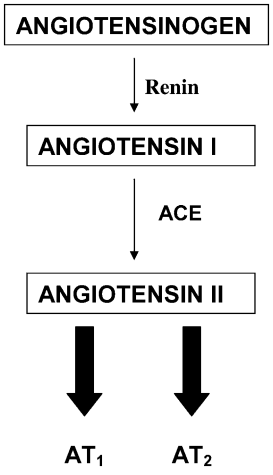

Fig. 1. Renin–angiotensin pathway (as represented in Herradon et al. [21]).

The results are summarized in Table 1. The results confirm that the expression levels of the genes of the renin–angiotensin pathway are profoundly influenced by *Mk* gene expression in mouse aorta and support the important conclusion that *Mk* gene expression has a critical role in the functions of the renin–angiotensin pathway and thus in the regulation of many important downstream effects of angiotensin II in aorta.

Comparison of the regulation of the renin–angiotensin pathway in Mk−/− vs. Ptn−/− mice

As previously demonstrated [21], the levels of expression of the genes of the renin–angiotensin pathway are also profoundly influenced by the levels of expression of the *Ptn* gene. To compare the influence of these structurally and functionally highly related family members, the levels of expression of the genes of the renin–angiotensin pathway in *Mk*−/− mouse aortae were compared with the levels of their expression of *Ptn*−/− mouse aortae (Table 1). The comparison makes it clear that both *Mk* and *Ptn* share regulation of critical elements in the normal development of this essential pathway and emphasize the remarkable collective importance of the PTN/MK developmental gene family in the regulation of the renin–angiotensin pathway in mouse aorta. However, the data also demonstrate a striking difference in the two pathways, since, in *Mk*−/− mouse aortae, an extraordinary increase of ~82-fold is seen in the levels of transcripts of angiotensinogen compared with levels in WT(+/+) mouse aortae, whereas no differences were seen in levels of transcripts of angiotensinogen in aorta in *Ptn*−/− mice compared with WT(+/+) mice.

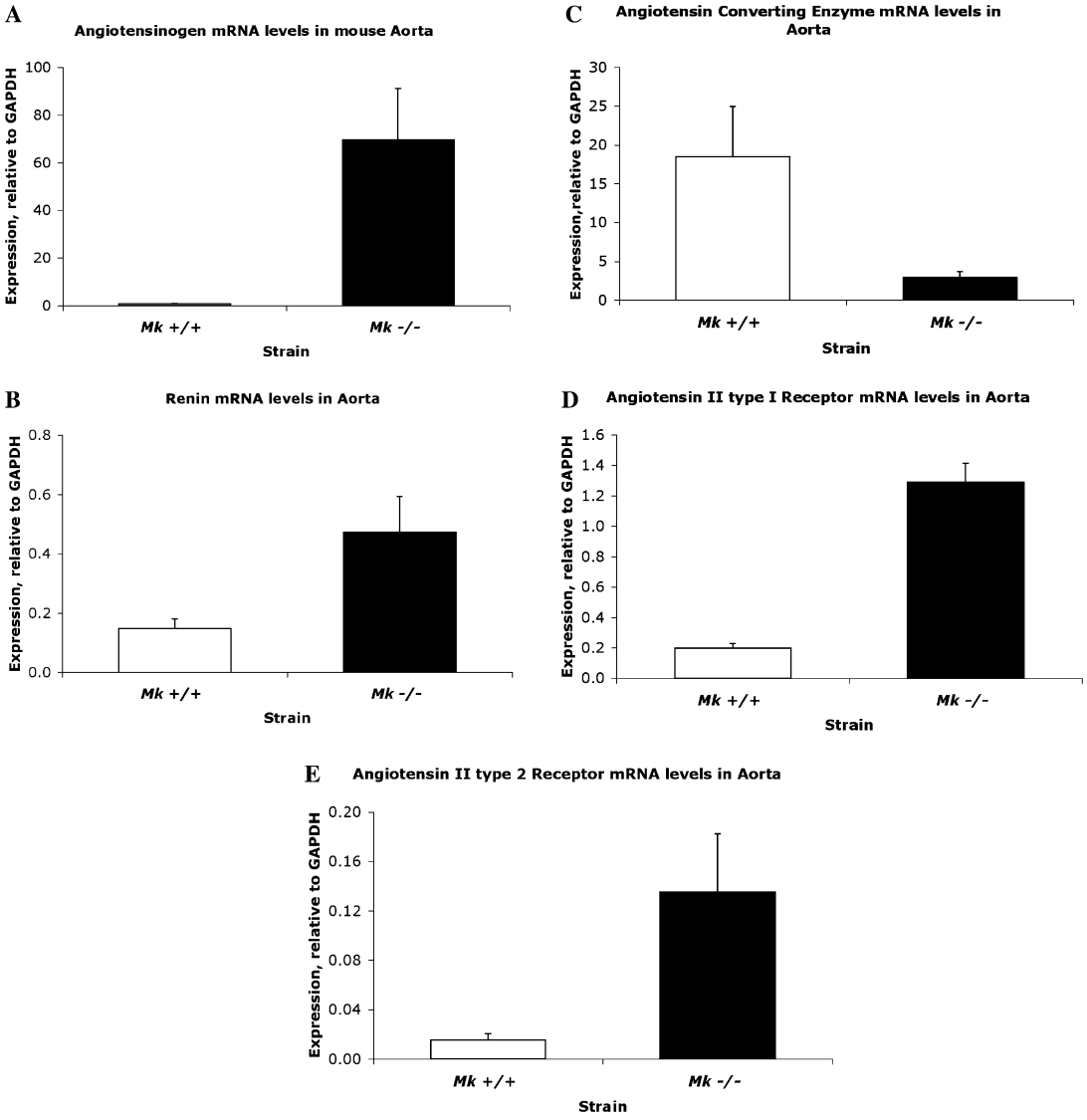

Fig. 2. Angiotensinogen (A), renin (B), angiotensin converting enzyme (C), angiotensin II type 1 (AT1) receptor (D), and angiotensin II type 2 (AT2) receptor (E) mRNA expression levels in aortae of *Mk*−/− and wild type WT(+/+) mice. Messenger RNA levels were measured by SYBR green RT-PCR and are expressed in relation to the levels of GAPDH used as a housekeeping gene. Aortae from three animals of each strain were used for RNA extraction and the determination was done on one only sample of each strain coming from the pool of the three different specimens. The results are shown as average ± SE of three different determinations.

Table 1
Expression levels of genes of the renin–angiotensin pathway in aortae of *Mk*−/− vs. WT(+/+) mice and *Ptn*−/− vs. WT(+/+) mice (Herradon et al. [21]

Gene	Aorta *Mk*−/− vs. WT(+/+)	Aorta *Ptn*−/− vs. WT(+/+)
Angiotensinogen	~82-fold ⇑	No difference
Renin	~3-fold ⇑	~1.5-fold ⇑
ACE	~6-fold ⇓	~3-fold ⇓
AT1 receptor	~6.5-fold ⇑	~5.5-fold ⇑
AT2 receptor	~9-fold ⇑	~6.2-fold ⇑

This table presents the comparison of the fold increase/decrease of the levels of the transcripts of the genes of the renin–angiotensin pathway in *Ptn*−/− mice vs. WT(+/+) mice (Herradon et al. [21]) and those above in *Mk*−/− mice vs. WT(+/+) mice.

Increased expression of the serine kinase (IKKα) inhibitor of IκB in aortae of Mk−/− mice: potential increased angiotensinogen expression levels in Mk−/− but not Ptn−/− mouse aortae through activation of NF-κB

To pursue a mechanism of the striking increase in angiotensinogen levels in *Mk*−/− mouse aortae that were not seen in aorta of *Ptn*−/− mice, we examined the levels of transcripts of the transcription factors known to specifically regulate the angiotensinogen gene in the microarray gene chips of *Ptn*−/−, *Mk*−/−, mice, and WT(+/+) mouse aortae. A significant increase in the levels of the mRNA of the serine kinase (IKKα), the serine kinase that phosphorylates and inactivates

Table 2
Expression levels of the kinase of inhibitor κB (IKKα) in aortae of *Mk*−/− vs. WT(+/+) mice and *Ptn*−/− vs. WT(+/+) mice

Gene	Aorta *Mk*−/− vs. WT(+/+)	Aorta *Ptn*−/− vs. WT(+/+)
Kinase of inhibitor κB (IKKα)	~2.5-fold ⇑	No difference

IκB, the inhibitor of nuclear factor kappa B (NF-κB) entry in nucleus [43], was found in *Mk*−/− mice (Table 2). Nuclear factor kappa B is a positive regulator of the levels of transcription of the angiotensinogen gene [44] and thus, the inactivation of IκB and the facilitated import of an activated NF-κB in *Mk*−/− mouse aortae suggest that NF-κB is a strong candidate to enhance transcription of angiotensinogen in *Mk*−/− mice, and furthermore, raise the possibility that NF-κB may have other important roles that are influenced by MK.

Discussion

The findings described in this manuscript demonstrate for the first time that MK regulates each of the genes encoding proteins unique to the renin–angiotensin pathway in mouse aorta; the results demonstrate directly that *Mk*−/− mouse aortae have highly significant increases in the levels of angiotensinogen, renin, and the angiotensin II type 1 and 2 receptors, and that *Mk*−/− aortae have decreased expression levels of ACE in comparison to aortae of WT(+/+) mice. We previously demonstrated that PTN equally regulates the renin–angiotensin pathway in mouse aorta [21]; similar to aortae of *Mk*−/− mice, *Ptn*−/− mouse aortae have sharp increases in the levels of the transcripts of renin and of the AT1 and AT2 receptors, and decreased levels of transcripts of ACE but, in *Ptn*−/− mouse aortae, an important exception is noted, since the expression levels of angiotensinogen do not differ from WT(+/+) mouse aortae in sharp distinction to the striking increase in levels of the angiotensinogen gene in *Mk*−/− mice.

The data thus demonstrate that the PTN/MK developmental gene family are major and most likely the major regulators of the renin–angiotensin pathway in mouse aorta during development. The highly significant difference of the extremely high level upregulation of the angiotensinogen gene in *Mk*−/− mice may be very important, since *Mk* gene expression peaks in mid-gestation and *Ptn* expression peaks in late gestation and in early neonatal growth. Thus, the renin–angiotensin pathway is differentially regulated during development by MK and PTN and thus the activities signaled by angiotensin II will differ at these times in the different cell types in which these two genes are most highly expressed. What the significance of activity of the pathways signaled by angiotensin II at the times of peak of *Ptn* and *Mk* expression remains unclear, since many of the structural proteins of aorta are not incorporated in aorta until later in neonatal life (Mecham et al., unpublished observations). It is anticipated that signaling through angiotensin II may be quite different in the different pathological conditions such as the human malignancies in which either *Mk* or *Ptn* genes are constitutively expressed.

The basis or the single differences represented by the dramatic increase in levels of the transcripts of angiotensinogen, the precursor of angiotensin II, is unlikely to be the result of the very low levels of ACE and the possible absence of feed back by angiotensin II, since ACE also is decreased in aorta of *Ptn*−/− mice [21]. However, the increased expression of IKKα, the essential activator of NF-κB needed for its import into nucleus [43,45] in *Mk*−/− mouse aortae (Table 2), suggests the possibility that increased NF-κB transcriptional activity as a result of its activation may be responsible for the high levels of expression of angiotensinogen in *Mk*−/− mice. Significant differences in the mRNA levels of the transcription factors known to regulate transcription of the different genes of the renin–angiotensin pathway themselves, including RelA:NF-κB1, c-jun, Hox, Sp1, and Sp3 [44,46–48], were not uncovered in the microarray data.

The data in this manuscript thus suggest that angiotensin II signaled phenotypes are important downstream consequences of levels of *Mk* expression and MK-signaling. Many of the known effects of MK are mimicked by angiotensin II; both MK and angiotensin II promote growth and glycosaminoglycan synthesis of vascular endothelial and smooth muscle cells [49,50], and both are potent angiogenic factors [13,25,51,52]. Midkine has important roles in renal development and is an important downstream mediator of the effects of retinoids in the developing kidney [53–55]. Each of the components of the renin–angiotensin pathway is highly expressed in the developing kidney in a pattern suggesting that angiotensin II is an important mediator of renal development [56]. Midkine also may be uniquely important in the development of heart and aorta [23] since high levels of *Mk* expression are found in the myocytes of embryonic heart [57] and during postnatal heart development [58], and MK stimulates the fibrinolytic activity of aortic endothelial cells [59,60], proliferation of aortic endothelial cells [50], and inflammation, since reductions in the migration of inflammatory leukocytes and osteoclast differentiation [61], and reduced capability to recruit neutrophils and macrophages to the arterial wall is found in *Mk*−/− mice [22,62].

These observations may be highly significant, since the renin–angiotensin pathway is subjected to control through existing therapeutics in circumstances where expression of *Mk* may be deregulated, such as the many human tumors with high level constitutive expression of *Mk* gene or rheumatoid arthritis, in which high level

expression of the *Mk* gene is found [63–69]. It is noted that antisense oligomers targeting midkine have been suggested for cancer therapies [40,70] whereas angiotensin converting enzyme inhibitors and specific antagonists of angiotensin II receptors have antiangiogenic and cytostatic effects [52,71,72].

We suggest that regulation of renin–angiotensin pathway by PTN/MK signaling and the ability of known therapeutics to regulate this pathway may present a much broader range of options in many different illnesses.

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Midkine regulates pleiotrophin organ-specific gene expression: Evidence for transcriptional regulation and functional redundancy within the pleiotrophin/midkine developmental gene family

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Abstract

Midkine (MK) and the highly related cytokine pleiotrophin (PTN) constitute the PTN/MK developmental gene family. The *Mk* and *Ptn* genes are essential for normal development of the catecholamine and renin–angiotensin pathways and the synthesis of different collagens. It is not known whether the *Ptn* and *Mk* genes regulate each other or whether PTN and MK are functionally redundant in development. We have now compared the levels of expression of *Ptn* and *Mk* in genetically deficient *Mk* −/− and *Ptn* −/− mice and found highly significant increases in *Ptn* gene expression in spinal cord, dorsal root ganglia, eye, heart, aorta, bladder, and urethra, but not in brain, bone marrow, testis, and lung of *Mk* −/− mice compared with wild type mice; a remarkable ~230-fold increase in *Ptn* expression levels was found in heart of *Mk* −/− mice and highly significant but lesser increases were found in six other organs. Differences in levels of *Mk* gene expression in *Ptn* −/− mice could not be detected in any of the organs tested. The data demonstrate that MK regulates *Ptn* gene expression with a high degree of organ specificity, suggesting that *Ptn* gene expression follows *Mk* gene expression in development, that the increase in *Ptn* gene expression is compensatory for the absence of MK in *Mk* −/− mice, that PTN and MK share a high degree of functional redundancy, and that MK may be very important in the development of heart in mouse.

Keywords: Pleiotrophin; Midkine; Development; Heart

The relationship of pleiotrophin (PTN the protein, *Ptn* the gene) [1,2] and midkine (MK the protein, *Mk* the gene) [3,4] was discovered when the *Ptn* gene was first cloned [2] and the predicted amino acid sequence of PTN was found to be over 50% identical to that of MK. Pleiotrophin, also known as the heparin binding growth-associated molecule (Hb-GAM) [5], together with MK, constitutes the PTN/MK developmental gene family. A critical question within this restricted family that has not been explained is whether *Ptn* and *Mk* expressions are important regulators of the levels of expression of each other and whether there is functional redundancy of PTN and MK during development.

Pleiotrophin gene expression levels are strictly regulated in temporal sequence and specificity of cell type during development and in inflammatory and other cells in normal wound healing [6–9] but, in adults, *Ptn* expression levels are stable and limited to only few cell types [10,11] unless increased in inflammatory and other

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cells in injured sites. The *Ptn* gene is a platelet-derived growth factor (PDGF)-inducible gene. The levels of *Ptn* expression peak 1–2 days after those of PDGF in late embryonic development and in the immediate postnatal period, the time of peaks of growth and differentiation in embryos [2,5]. Upregulation of *Ptn* in cells at injured sites also follows PDGF upregulation by 1–2 days, a result consistent with the putative role of PTN as a downstream-signaling molecule of PDGF [12,13]. The 5′ promoter region of the *Ptn* gene has a functional serum response element to account for the PDGF-stimulated upregulation of *Ptn* gene expression [13], establishing further that PTN is a member of the PDGF-inducible gene family.

In contrast, *Mk* gene expression is not influenced in cells stimulated by PDGF; *Mk* gene expression is increased in mouse embryonic carcinoma cells treated with retinoic acid and *Mk* gene expression levels correlate with early stages of retinoic acid-induced differentiation in the mid-gestation period of mouse embryogenesis [14–17]; thus, the *Mk* gene is expressed earlier in development than the *Ptn* gene but the sites of its expression significantly, but not completely, overlap with those of *Ptn* [18,19]. In adults, like *Ptn*, *Mk* gene expression is limited to only few cell types [14] and also is upregulated in inflammatory and other cells after injury [20].

Pleiotrophin is a 136 amino acid heparin-binding cytokine [1,2], midkine is a 121 amino acid cytokine that also binds heparin [21]. Both *Ptn* and *Mk* encode signal peptide containing proteins with lysine rich domains at the N- and C-termini [2] and have significant homology in each of two separate heparin-binding thrombospondin type I repeat-like domains [22,23]. Each of the two thrombospondin type I repeat-like domains in both PTN and MK signals different phenotypes than the other when they are expressed independently of each other and thus are likely to recognize different receptor-like proteins [24]. However, there is functional overlap in the signals initiated by each of the functional domains in PTN when compared with MK.

Pleiotrophin and MK stimulate proliferation of different, but overlapping, cell types in culture [1,2,6,24–28]. Both PTN and MK also stimulate neurite outgrowth from primary cultures of neonatal neuronal cells [1,2,5,29] and angiogenesis in vivo [6,24,25,28,30,31] (Zhang et al., in progress). Pleiotrophin stimulates lineage-specific differentiation of glial progenitor cells (Yeh et al., in progress) and MK promotes the survival of embryonic neurons [15,32]. Midkine enhances the fibrinolytic activity of the aortic endothelial cells [33,34] and may be uniquely important in the development of heart; it is strongly expressed in the myocytes of embryonic heart [35] and highly regulated during postnatal heart development [36]. It also stimulates the fibrinolytic activity of aortic endothelial cells [33,34] and proliferation of aortic endothelial cells, an activity that is dependent on its activity in neighboring smooth muscle cells [37]. Importantly also, mice genetically deficient in *Mk* have a significantly reduced neointima formation in restenosis injury models that is reversible by systemic MK administration [38] and antisense *Mk* oligodeoxyribonucleotides suppress neointima formation in vivo [39], suggesting that *Mk* gene expression may be crucial also in aorta and suggesting the conclusion that both MK and PTN function in diverse, but overlapping, ways; MK and PTN thus appear to signal both proliferation and differentiation responses dependent on the different cellular contexts in which they are expressed.

Pleiotrophin and MK are also important in the malignant phenotype of different cancer cells; both the *Ptn* and *Mk* genes transform non-transformed cells [24,40–42] and activated *Ptn* and *Mk* gene expression is found in many human tumors of diverse origins [3,41,43–49]. Initiation of constitutive *Ptn* and *Mk* gene expression through acquisition of an activated *Ptn* gene "switches" the premalignant cells to cells with a higher stage of malignancy; the properties of the cells that have acquired the activated *Ptn* gene are the same properties of the malignant cells with activated endogenous *Ptn* and *Mk* genes, signifying that both *Ptn* and *Mk* function as a potent tumor promoter in vivo when activated by mutation in the premalignant cell.

Pleiotrophin signals through inactivation of its receptor, the transmembrane receptor protein tyrosine phosphatase (RPTP) β/ζ, through a presumed conformational change that blocks access of substrate to the active site of RPTPβ/ζ. The inactivation of RPTPβ/ζ initiates increased levels of tyrosine phosphorylation of the RPTPβ/ζ substrates, including β-catenin [50], β-adducin (Pariser et al., submitted) and Fyn [51], due to the persistent activity of a protein kinase(s) acting in balance with the tyrosine phosphatase activity of RPTPβ/ζ in cells that are not stimulated with PTN. Less is known of the MK-signaling pathways, although reports have suggested that PTN and MK may share in common receptors such as RPTPβ/ζ [52], syndecan-3 [23], and the anaplastic lymphoma kinase (Alk) [27,53].

These different findings cited above make clear both the many overlapping functions of PTN and MK, and the different phenotypes signaled by PTN and MK. In these experiments, we demonstrate that MK regulates *Ptn* gene transcription; highly significant increases in *Ptn* gene expression are demonstrated in specific organs in *Mk* −/− mice compared with *Ptn* gene expression levels in WT (+/+) mice, suggesting not only that *Mk* is upstream of *Ptn* and thus "cross talk" within the PTN/MK developmental gene family but the increase in expression of *Ptn* in *Mk* −/− mice is compensatory for the absence of *Mk* and thus PTN is functionally redundant and likely to initiate pathways highly related, possibly identical to MK-signaling pathways in *Mk* −/−

mice. The data thus provide the first evidence to establish that *Mk* gene expression levels critically regulate the levels of *Ptn* gene expression in organs that depend on *Mk* gene expression during development.

Materials and methods

Genotyping of Ptn −/− and Mk −/− mice. The *Mk* −/− mice were generated by methods essentially identical to those previously described [54]. The animals used in this study were male *Mk* −/− and WT (+/+) mice at 8 weeks of age. The assays were carried out in accordance with the NIH guidelines for Care and Use of Laboratory Animals.

The genotypes of the *Mk* −/− mice were confirmed prior to sacrifice with the polymerase chain reaction using as primers 5′-ATC GGT TCC AAG TCC TCC CTC CGT C-3′ forward and 5′-CAC CTT CCT CAG TTG ACA AAG ACA AGC-3′ reverse to generate from genomic DNA extracted from tails of *Mk* −/− and WT (+/+) mice a cDNA of ~0.7 kb.

We also used *Ptn* −/− mice generated as previously described [55,56]. The genotypes of *Ptn* −/− mice were confirmed by polymerase chain reaction using as primers 5′-GAT TGA ACA AGA TGG ATT GC-3′ forward and 5′-CAT TTA GGC AAA CAG GAA GGA CG-3′ reverse to generate a cDNA of ~0.7 kb detected in agarose gels from genomic DNA extracted from tails of *Ptn* −/− and WT (+/+) mice.

Tissue acquisition, RNA extraction, and gene chip analysis. After being anaesthetized with halothane, *Mk* −/−, *Ptn* −/−, and WT (+/+) mice were sacrificed. Eleven different tissues (brain, spinal cord, dorsal root ganglia, eye, heart, aorta, lung, urethra, bladder, testis, and bone marrow) were rapidly dissected from 3 animals per strain, frozen in dry ice, and stored at −80 °C before RNA isolation. Frozen tissues were homogenized in 1 ml of the TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50–100 mg tissue and total RNA was extracted following the manufacturer's protocol. The concentrations of RNA in each sample were measured by A_{260} and the integrity of RNA was confirmed in 1.25% agarose gels after electrophoresis. RNA samples were treated with a preparation of DNAses (Ambion, Austin, TX) following the manufacturer's protocol. Pooled RNA from three samples of each strain was used for microarray analysis.

Affymetrix mouse 14,400 oligonucleotide probe Genome MOE 430A Gene Chips were used according to standard protocols supplied by the manufacturer, as we described previously [56]. Different "housekeeping" genes, including actin, GAPDH, and hexokinase, and other control sequences were included as reference and quality control indicators.

cDNA synthesis and SYBR green RT-PCR analysis. Complementary DNAs were synthesized from the pool of three samples of total RNA of all tissues from WT (+/+), *Ptn* −/−, and *Mk* −/− mice using a cDNA synthesis kit (Bio-Rad, Hercules, CA). The SYBR green RT-PCR method (Bio-Rad, Hercules, CA) was used to confirm results obtained in the gene chip studies using the following primer sets (forward and reverse): pleiotrophin (5′-TTGGGGAGAATGTGACCTCAATAC-3′, 5′-GGCTTGGAGATGGTGACAGTTTTC-3′); GAPDH (5′-CCTG CACCACCAACTGCTTA-3′, 5′-TCATGAGCCCTTCCACAATG-3′) [57]; midkine (5′-AAACCGAACTCCAGGACCAGAGAC-3′; 5′-A ACACTCGCTGCCCTTCTTCAC-3′). As a negative control for the correctness of the primers used in real time RT-PCR of the *Ptn* gene, we tested them with cDNA from *Ptn* −/− mice and demonstrated the absence of *Ptn* gene expression (data not shown). The relative expression of each gene was normalized against GAPDH, the reference standard, as described by the manufacturer's user bulletin # 2 of ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Other reference standards were tested and found to be less stable in the experiments testing WT (+/+) and *Mk* −/− mice.

Results

Pleiotrophin gene expression is markedly increased in many, but not all, organs in midkine −/− mice

Using the methods and data derived from Affymetrix chip microarrays from WT (+/+) mice and mice genetically deficient in the *Mk* gene (*Mk* −/−) described above, we compared the levels of *Ptn* gene expression in 11 organs in WT (+/+) mice with its level of expression in *Mk* −/− mice. Highly significant increases in the levels of *Ptn* gene expression were demonstrated in 7 of the 11 tissues tested from *Mk* −/− mice compared to their levels of expression in WT (+/+); spinal cord, dorsal root ganglia (DRG), eye, heart, aorta, bladder, and urethra each have highly significant increases in the levels of *Ptn* gene expression in *Mk*−/− mice compared with levels of *Ptn* gene expression in WT (+/+) mice. In contrast, brain, bone marrow, testis, and lung of *Mk* −/− mice levels of *Ptn* gene expression were not detectably different from those in WT (+/+) mice (data not shown).

To confirm and pursue the significance of the differences in the levels of the *Ptn* gene identified in the microarray analysis above, triplicate determinations of expression levels of the *Ptn* gene were measured in each of the organs identified above, using the SYBR green RT-PCR methodology. The more sensitive SYBR green RT-PCR technology directly confirmed the results obtained in the microarray analysis; highly significant increases in the levels of the transcripts of the *Ptn* gene were observed in spinal cord, dorsal root ganglia, eye, heart, aorta, bladder, and urethra of *Mk* −/− mice compared with WT (+/+) mice (Figs. 1A–G, summarized in Table 1). As a control, the levels of *Ptn* mRNA in *Ptn* −/− mice were determined in each of the 11 tissues studied above and found to be absent in these same tissues. Levels of GAPDH were used as standard to express relative levels of *Ptn* gene expression in each of the organs studied from *Mk* −/− mice.

The data clearly establish that *Ptn* gene expression is selectively and strikingly elevated in many, but not all, organs in *Mk* −/− mice. The data also point to the very striking increases in gene expression in heart of *Mk* −/− mice; an ~230-fold increase in *Ptn* gene expression was found. Lesser, but highly significant, increases in *Ptn* gene expression also were found in eye (~10.5-fold increase), aorta (~8.5-fold increase), urethra (~7-fold increase), spinal cord (~5-fold increase), dorsal root ganglia (~2.5-fold increase), and bladder (~2.5-fold increase) in *Mk* −/− mice in comparison to WT (+/+) mice. The strikingly high level *Ptn* gene expression in heart and to a lesser, but significant, degree in aorta, urethra, spinal cord, dorsal root ganglia, and bladder point to the conclusion that upregulated *Ptn* gene expression serves to compensate in part for the absence

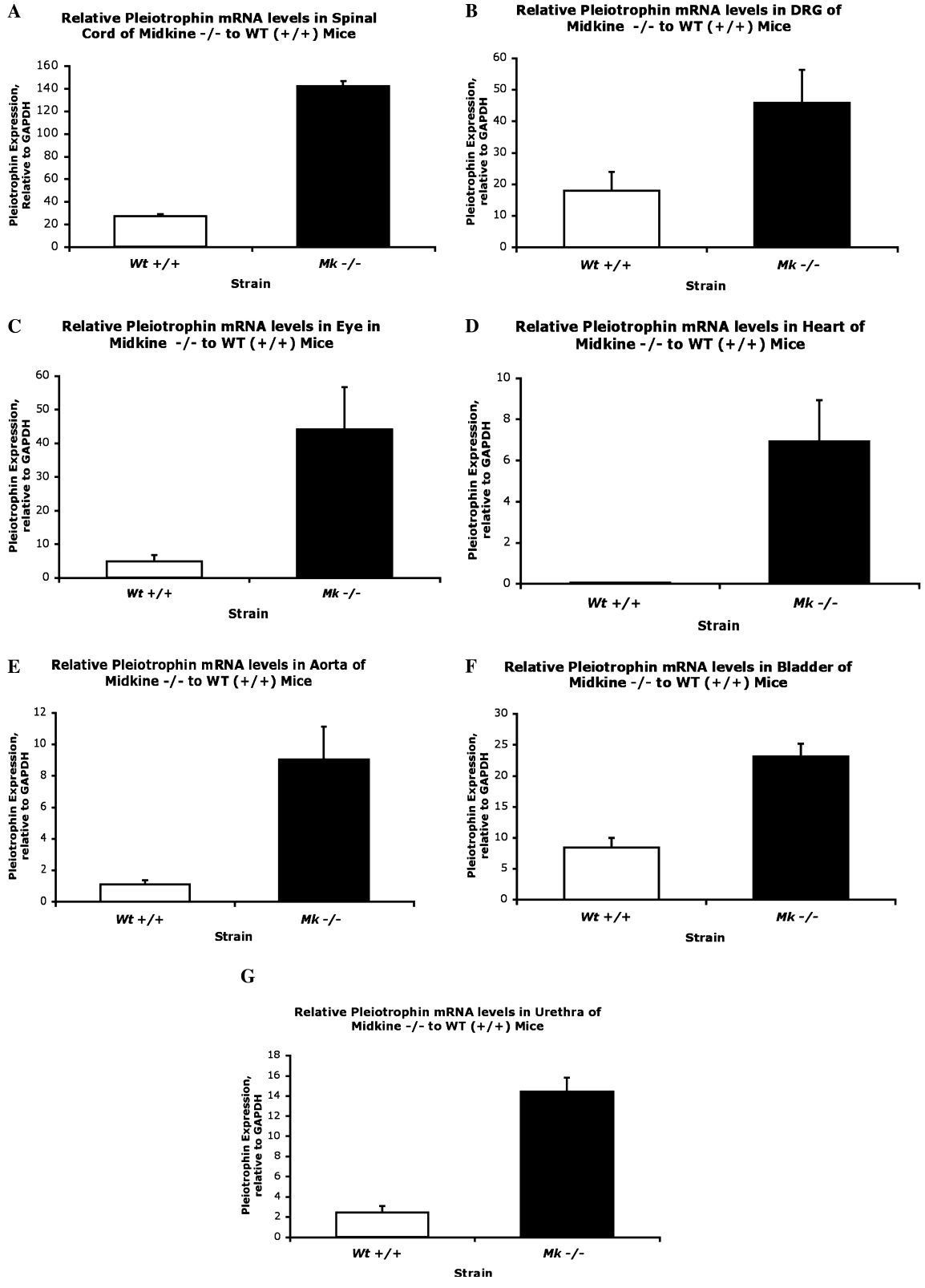

Fig. 1. Pleiotrophin mRNA expression levels in (A) spinal cord, (B) dorsal root ganglia, (C) eye, (D) heart, (E) aorta, (F) bladder, and (G) urethra of *Mk* −/− and WT (+/+) mice. Pleiotrophin mRNA levels were measured by SYBR green RT-PCR and are expressed as the ratio of levels of *Ptn* mRNA relative to levels of GAPDH mRNA used as a control housekeeping gene. Tissues from three animals of each strain were used for RNA extraction and the determination was done from one sample of each strain derived from the pool of the three different specimens. The results shown are the average ± SE of each of three different determinations.

Table 1
Upregulation of *Ptn* gene expression levels in different organs of *Mk* −/− vs. WT (+/+) mice

Tissue	*Mk* −/− vs. WT (+/+)
Heart	230-fold↑
Eye	10.5-fold↑
Aorta	8.5-fold↑
Urethra	7-fold↑
Spinal cord	5-fold↑
Dorsal root ganglia	2.5-fold↑
Bladder	2.5-fold↑
Brain	No difference
Lung	No difference
Testis	No difference
Bone marrow	No difference

The table summarizes data represented in Fig. 1 in which *Ptn* gene expression levels were measured by SYBR green RT-PCR in different organs of *Mk* −/− compared to WT (+/+).

of *Mk* and thus suggests functional redundancy of PTN- and MK-signaling in specific mouse organs. The very high levels of *Ptn* expressed in heart of *Mk* −/− mice also suggest the major need and thus high importance of MK-signaling in these organs during development.

Comparison of expression levels of the midkine gene in different tissues of Ptn −/− mice and WT (+/+)

We also compared *Mk* gene expression in the same 11 different organs studied above in *Ptn* −/− and WT (+/+) mice. In contrast to *Ptn* gene expression in *Mk* −/− mice, no differences in the levels of expression of *Mk* were seen in any of the organs in which *Mk* gene expression levels were compared in *Ptn* −/− and WT (+/+) mice (data not shown). These data were confirmed by SYBR green RT-PCR, and again, no differences were seen in any of the organs in which *Mk* gene expression levels were compared in *Ptn* −/− and WT (+/+) mice.

Discussion

The data described in this manuscript demonstrate for first time that the proteins encoded by the PTN/MK developmental gene family are not only structurally and functionally related but regulated together at the level of transcription as well. The results demonstrate that *Mk* −/− mice have highly significant increases in the levels of *Ptn* mRNA in the spinal cord, DRG, eye, heart, aorta, bladder, and urethra, but not in brain, bone marrow, testis, and lung compared with the levels of *Ptn* mRNA expressed in WT (+/+) mice. The levels of *Mk* gene expression are not different in *Ptn* −/− mice when compared with levels of *Mk* in WT (+/+) mice. Since upregulated *Ptn* gene expression follows upregulation of *Mk* gene expression by 1–2 days in mouse embryos during development and in cells at sites of injury, the increase in *Ptn* expression in *Mk* −/− mice indicates that *Ptn* expression is transcriptionally regulated through levels of *Mk* expression and suggest that PTN functions downstream of MK in development and compensates for the absence of *Mk* gene expression in *Mk* −/− mice through functional redundancy. The demonstrations of Nakamura et al. [54] and of Bronson et al. (unpublished observations) that *Mk* −/− mice lack evident gross or histological abnormalities support the hypothesis that the important functions of MK during development have been sufficiently compensated by upregulated levels of *Ptn* gene expression in *Mk* −/− mice. Thus, based on overlapping functions in vitro and the temporal sequence of upregulated *Mk* and *Ptn* gene expression during development [6–11], it is concluded that a compensatory increase in *Ptn* transcription signaled by the absence of *Mk* gene expression serves to effectively initiate signals through PTN that are normally initiated by MK and thus PTN is functionally redundant with MK during development. The extremely high levels of *Ptn* gene expression in heart and other organs such as aorta likely reflect a high level of importance to MK-signaling in heart and aorta and thus the degree of need for MK in these organs for functional differentiation of different pathways during development.

Previous reports support this conclusion. Both *Mk* and *Ptn* are likely to be important in postnatal development in mouse heart [36]. Both *Ptn* and *Mk* genes are upregulated in myocardial infarction and ischemic myocardium [35,58,59]. Among the pathways critically regulated by both *Mk* and *Ptn* are the catecholamine biosynthesis pathway [56] (Herradon et al., in preparation) and the renin–angiotensin system [60] (Ezquerra et al., submitted), two pathways essential to regulate the cardiovascular system and angiogenesis. Thus, the very high levels of *Ptn* gene expression in heart and aorta of *Mk* −/− mice may compensate for the absence of *Mk* gene expression to support the vital pathways of catecholamine biosynthesis and the renin–angiotensin pathway but subtle differences in these pathways signaled by MK and PTN (Herradon et al., submitted) alter the full extent of the functional redundancy of PTN to be proved for MK in functions of heart. The hypothesis that upregulated PTN-signaling in heart is in major compensation for lack of *Mk* gene expression is predictive of severe dysfunction of both heart and aorta in the *Mk* −/−, *Ptn* −/− double gene deletion mice, since heart lacks both PTN- and MK-signaling during development, and perhaps also in the different pathological conditions in which *Mk* or *Ptn* gene expression levels are elevated.

As noted earlier, both MK and PTN initiate and maintain the differentiated state of neuronal cells [1,2,5,15,16,61–63] and *Mk* −/− mice display delayed hippocampal development [54]. Pleiotrophin also is involved in hippocampal synaptic plasticity and regulation of learning-related behavior and suppresses long-term

potentiation of hippocampus [55,64,65]. The increased expression of *Ptn* in DRG and spinal cord of *Mk* −/− mice coupled with the failure to identify gross morphological abnormalities in DRG and spinal cord thus suggest that PTN compensates for the absence of *Mk* in these organs and thus also suggest functional redundancy of MK and PTN in vivo. However, significant increases in *Ptn* gene expression levels were not seen in brain. In the case of brain the large mass of brain tissue used to purify mRNA may have precluded detection of upregulated *Ptn* gene expression in the specific nuclei and sites in *Mk* −/− mice that serve these important functional roles.

The data also present the first evidence that PTN and MK may have important roles in development of eye, bladder, and urethra; each of these organs was found to have significant increases in levels of *Ptn* gene expression in *Mk* −/− mice. Both MK and PTN have been identified in the embryonic urogenital system, including ureter [19], but not in urethra or bladder, although both *Ptn* and *Mk* genes are highly expressed in malignant bladder tumors raising the possibility that PTN- and/or MK-signaling may be very important in the malignant phenotype of these malignant bladder tumors [66–68]. The *Ptn* and *Mk* genes are known to be expressed in normal eyes [69,70] (Ezquerra et al., 2004 unpublished observations) and increased levels of *Mk* expression have been identified in retinal ischemia [71] and of *Ptn* in corneal neovascularization [72].

It is possible to speculate that other factors also are likely to be affected by the absence of *Ptn* or *Mk* expression in heart; for example, *Mk* is expressed in the myocytes and endocardium of the adult rat heart [35]. Its pattern of expression is similar to that of the basic (b) fibroblast growth factor (FGF) [73]. Since bFGF promotes survival and regulates migration of adult myocytes and the production and constitution of extracellular matrix [35], overlapping functions with both PTN and MK (unpublished), it is possible that *Mk* expression levels may also regulate bFGF gene expression and bFGF may also be in part functionally redundant with MK.

In summary, the data establish for the first time that MK regulates transcription levels of the *Ptn* gene in an organ-specific manner; through the striking increase in *Ptn* expression in *Mk* −/− mice, it is suggested that MK and PTN are functionally redundant, and that upregulated *Ptn* gene expression is compensatory for deficient MK function, providing strong support for the functional importance of the PTN/MK developmental gene family in mouse development.

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Pleiotrophin stimulates tyrosine phosphorylation of β-adducin through inactivation of the transmembrane receptor protein tyrosine phosphatase β/ζ

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Abstract

Pleiotrophin (PTN the protein, *Ptn* the gene) signals through a unique mechanism; it inactivates the tyrosine phosphatase activity of its receptor, the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ, and increases tyrosine phosphorylation of the substrates of RPTPβ/ζ through the continued activity of a yet to be described protein tyrosine kinase(s) in PTN-stimulated cells. We have now found that the cytoskeletal protein β-adducin interacts with the intracellular domain of RPTPβ/ζ in a yeast two-hybrid system, that β-adducin is a substrate of RPTPβ/ζ, that β-adducin is phosphorylated in tyrosine in cells not stimulated by PTN, and that tyrosine phosphorylation of β-adducin is sharply increased in PTN-stimulated cells, suggesting that β-adducin is a downstream target of and regulated by the PTN/RPTPβ/ζ signaling pathway. β-Catenin was the first downstream target of the PTN/RPTPβ/ζ signaling pathway to be identified; these data thus also suggest that PTN coordinately regulates steady state levels of tyrosine phosphorylation of the important cytoskeletal proteins β-adducin and β-catenin and, through PTN-stimulated tyrosine phosphorylation, β-adducin may contribute to the disruption of cytoskeletal structure, increased plasticity, and loss of homophilic cell–cell adhesion that are the consequences of PTN stimulation of cells and a characteristic feature of different malignant cells with mutations that activate constitutive expression of the endogenous *Ptn* gene.

Keywords: Pleiotrophin; β-Adducin; Receptor protein tyrosine phosphatase β/ζ

Pleiotrophin (PTN the protein, *Ptn* the gene) is a highly conserved 136 amino acid growth promoting, differentiation inducing cytokine [1–4]. Pleiotrophin expression is important in different developmental pathways [5,6], responses to injury [7], and in the progression of many human tumors [8–10] (Chang et al., Submitted; Perez-Pinera et al. Submitted).

Pleiotrophin is over 50% identical in amino acid sequence with midkine (MK the protein, *Mk* the gene), the only other member of *Ptn*/*Mk* developmental gene family [3,8,11]. Its expression is extensively regulated in embryonic development in a temporal and cell type-specific manner primarily in nervous and vascular systems [1,3,4,7,10,12–14]. However *Ptn* gene expression is constitutive and limited to few cell types in adults unless it is up-regulated in inflammatory macrophages, fibroblasts, and endothelial and other cells at sites of injury [7].

Pleiotrophin stimulates diverse functional responses in different contexts. It stimulates proliferation of fibroblasts, endothelial cells, and epithelial cells in culture [1,3,10,15,16], and neurite outgrowth and differentiation responses in neonatal neuronal cells [1,2] and both

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endogenous PTN and exogenous stimulate process outgrowth and oligodendrocyte lineage specific differentiation responses in glial progenitor cells in primary culture [7,17]. Male mice into which a dominant negative *Ptn* gene has been introduced have striking apoptosis in early sperm development and Sertoli cells [18] and recently, *Ptn* gene expression has been demonstrated to be essential for synthesis of the enzymes of the catecholamine biosynthesis pathway [5] and of the different proteins of the renin–angiotensin pathway [6,19].

The *Ptn* gene also is a proto-oncogene [9]. Mutations that activate constitutive *Ptn* gene expression "switch" the phenotype of pre-malignant cells to a phenotype of high malignancy, a phenotype often associated with striking tumor angiogenesis (Chang, et al. Submitted) [8,10]. Furthermore, mutations that activate the endogenous *Ptn* and establish constitutive *Ptn* gene expression are frequent in many human tumors of different histological origins [20–24]. When PTN signaling is interrupted by a dominant negative inhibitor of PTN [21] (Chang et al., Submitted) or with ribozyme constructions [25,26], the malignant phenotype of these highly malignant cells reverts to the pre-malignant phenotype. Collectively, the data strongly support the conclusion that activating mutations of the endogenous *Ptn* gene in the premalignant cell not only are frequent but alone are sufficient to initiate the highly malignant phenotype in many human cancers; mutations activating the *Ptn* gene may be among the most important in the progression of human malignancies.

The studies in the manuscript were designed to pursue the mechanisms by which PTN signals many different cellular responses noted above. In previous studies, we demonstrated that the transmembrane receptor protein tyrosine phosphatase (RPTP)β/ζ (Fig. 1) transmits the PTN signal; this pathway of PTN signaling is unique, since PTN is the first natural ligand to be discovered for any of the transmembrane tyrosine phosphatases [27]. RPTPβ/ζ is an important protein that is believed to have diverse function; it has numerous chondroitin sulfate side chains, and is expressed predominantly in brain [28–30]. The extracellular domain of RPTPβ/ζ differs significantly from other RPTPs. It has a carbonic anhydrase domain at the extreme N-terminal region, a fibronectin type III domain, and "cysteine free" domain immediately before the transmembrane spanning domain. RPTPβ/ζ binds different extracellular matrix molecules [31,32] and binds to and presumably is the receptor recognizing the *Helicobacter pylori* toxin Vac A [33], but, whether Vac A initiates a downstream signal is not known. There are three alternative splice variants of RPTPβ/ζ [34], including a full-length transmembrane form, a short transmembrane form lacking the extracellular, membrane proximal "cysteine free" domain, and a soluble form containing essentially the entire extracellular domain known as 6B4 proteoglycan, or "phosphacan" [35]. The intracellular domain of RPTPβ/ζ is highly conserved with the intracellular domain of other transmembrane receptor protein tyrosine phosphatases. The proximal intracellular domain of RPTPβ/ζ (defined here as the D1 domain, residues 1663–2034, Fig. 1) contains the active tyrosine phosphatase site of RPTPβ/ζ and the catalytic cysteine required for tyrosine phosphatase activity is residue 1932. The membrane distal (D2) domain contains a second, but inactive, tyrosine phosphatase domain and the extreme C-terminal amino acids define a short hydrophobic sequence predicted to bind to PDZ domain-containing proteins [36]. It was shown previously that PTN enforces dimerization of RPTPβ/ζ and inactivates its catalytic activity [27], presumably through a conformational change in the D1 (active site containing) domain of RPTPβ/ζ that prevents substrates phosphorylated in tyrosine from gaining access to their active site; this mechanism of the conformational change and block of access to the active site was demonstrated when the crystal structure of RPTPα was first solved [37]. PTN is the first naturally occurring ligand to any of the RPTPs and thus the PTN/MK signaling pathway is unique.

β-catenin was the first substrate of RPTPβ/ζ to be discovered. Tyrosine phosphorylation of β-catenin is markedly increased in PTN-stimulated cells [27] and the increase in tyrosine phosphorylation of β-catenin in PTN-stimulated cells reduces its affinity for the cytoplasmic tails of the cadherins (Perez-Pinera et al., submitted) disrupts the link of the cadherins and β-cate-

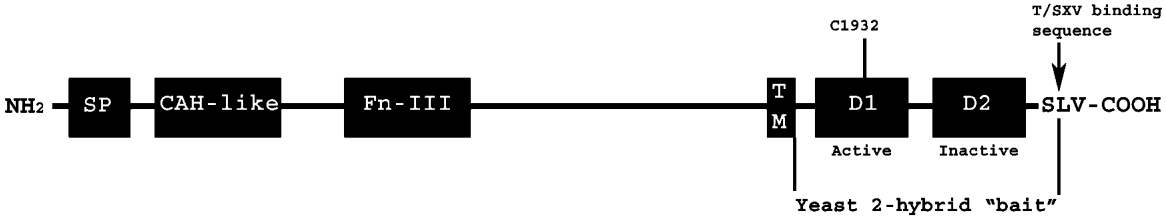

Fig. 1. Schematic representation of receptor protein tyrosine phosphatase (RPTP)β/ζ, SP, signal peptide; CAH-like, carbonic anhydrase-like domain; Fn-III, fibronectin type III domain containing condroitin sulfate; TM, transmembrane domain; D1, active tyrosine phosphatase domain (C1932-phosphatase catalytic residue); D2, inactive tyrosine phosphatase domain; SLV, C-terminal PDZ binding sequence. Yeast two-hybrid "bait", residues 1663–2314.

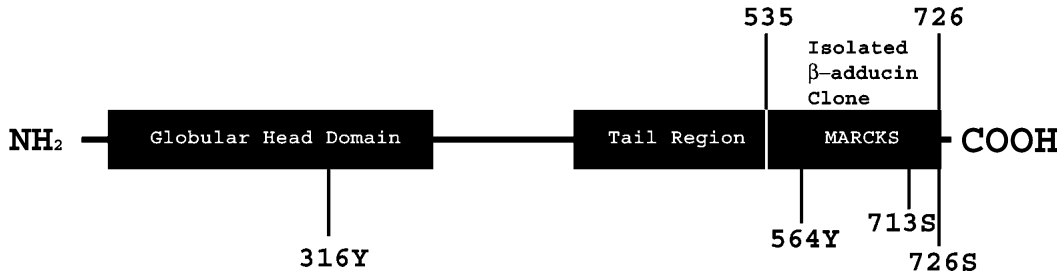

Fig. 2. Schematic representation of β-adducin. Shown is the globular head domain involved in the β-adducin multimer binding, the tail region containing the interactive clone of β-adducin identified in the yeast two-hybrid screen (residues 535–726), the myristoylated alanine-rich C-kinase substrate-like domain (MARCKS), and the consensus protein kinase C sites (residues 713S, and 726S), and the single putative tyrosine phosphorylation sites (residue 564Y).

nin through α-catenin with actin, disrupts adherent junctions, and reduces homophilic cell–cell adhesion (Perez-Pinera et al., submitted). These features are the early responses in PTN-stimulated cells [27] and are consistent with the known properties of β-catenin phosphorylated in tyrosines to disrupt these vital cytoskeletal complexes [38–41].

This study aimed to identify additional proteins interactive with RPTPβ/ζ and thus additional targets of the PTN/RPTPβ/ζ intracellular signaling cascade. The yeast two-hybrid system was used since it is a well-established and powerful tool to identify molecular interactions of proteins [42]. We screened a human fetal brain cDNA expression library with the intracellular domain of RPTPβ/ζ and identified the C-terminal 191 amino acids of β-adducin (see Fig. 2). β-adducin, like β-catenin, is an integral protein in cytoskeletal structure, and an important regulator of the integrity and fluidity of the cytoskeleton [43–47]. The association of the 191 amino acids fragment of β-adducin with the cytoplasmic domain of RPTPβ/ζ, therefore, suggested that β-adducin may be regulated coordinately by the PTN/RPTPβ/ζ signaling pathway and potentially β-adducin is a downstream target of PTN. The data generated now demonstrate directly that β-adducin is regulated through the PTN/RPTPβ/ζ signaling pathway.

Materials and methods

Yeast two-hybrid screen. AH109 competent yeast cells, the yeast expression vectors pGBKT7 and pACT1, and the yeast cells Y187 pretransformed human fetal brain MATCHMAKER cDNA Library were obtained from BD Biosciences Clontech (La Jolla, CA). The human fetal brain library was selected because of the previously observed high-level expression of RPTPβ/ζ and PTN in early brain development [12], suggesting that proteins in the PTN signaling pathway may be expressed in high levels in brain at that time. The full-length RPTPβ/ζ clone (Fig. 1) (GenBank Accession No. NM_002851) was a generous gift from H. Saito, Dana Farber Cancer Center, Boston, MA. The cytoplasmic domain of RPTPβ/ζ (residues 1663–2314) (see Fig. 1) was amplified using polymerase chain reaction (PCR) and subcloned into the yeast expression vector pGBKT7 for transformation of AH109 competent yeast cells. AH109 cells expressing RPTPβ/ζ residues 1663–2314 were confirmed by DNA sequencing analysis and Western blots of the induced AH109 cell lysates probed with anti-RPTPβ/ζ- specific antibodies (BD Transduction Laboratory, San Diego, CA). The confirmed strain was co-cultured with compatible Y187 yeast cells containing the pre-transformed human fetal brain library overnight in a shaker at 30 °C in 50 ml of YPAD yeast media. The mating yeast were plated on SD medium-stringency selection plates (SD/-His/-Leu/-Trp) and surviving colonies were re-plated on high stringency SD medium (SD/-His/-Leu/-Trp/-Ade). The colonies stained blue were tested for β-galactosidase activity with a colony filter-lift assay, the isolated library clones confirmed to be positive were purified, the cDNAs were sequenced, and the encoded proteins were identified by screening different standard databases.

To confirm the interaction of the isolated clones with the cytoplasmic domain of RPTPβ/ζ, AH109 cells were co-transformed with the vector pGBKT7-RPTPβ/ζ cytoplasmic domain and the vector pACT2 containing the brain library clone whose interaction was characterized as above. The isolated clones were confirmed for their ability to grow on the highest stringency selection plates and expression of α- and β-galactosidases.

Preparation of glutathione-S-transferase fusion proteins. The active site containing D1 domain of RPTPβ/ζ (residues 1663–2034) and an inactivated active site RPTPβ/ζ D1 domain mutant (residues 1663–2034, C1932S) were prepared by inserting the cDNA fragment encoding the human RPTPβ/ζ, amino acids 1663–2034 or 1663–2034 (C1932S) fused with glutathione-*S*-transferase (GST) to the bacterial expression plasmid pGEX-KG (Amersham Pharmacia, Piscataway, NJ). The GST fusion proteins have been termed GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932), respectively. The constructs (or GST alone) were expressed in BL-21 competent cells grown in 100 ml Luria–Bertani broth overnight, grown to middle log phase, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h, and lysed with 1% Triton X-100 in PBS with 0.1% phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, and 1× Complete Protease Inhibition Cocktail (Roche Applied Sciences, Indianapolis, IN). The GST and GST fusion proteins were immobilized on 100 μl of glutathione–Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ) and washed with 1% Triton X-100 in PBS. The GST-RPTPβ/ζ D1 fusion protein activity was tested by measuring its ability to dephosphorylate β-catenin phosphorylated in tyrosine to confirm it is an active tyrosine phosphatase. GST-RPTPβ/ζ D1 (C1932S) was shown to bind to, but not to dephosphorylate, β-catenin phosphorylated in tyrosine as previously described [27].

RPTPβ/ζ D1 and D1 (C1932S) GST "Capture" of β-adducin from cell lysates. To "capture" proteins interactive with RPTPβ/ζ D1 or RPTPβ/ζ D1 (C1932S), HeLa cell lysates were prepared and incubated at 4 °C with GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S) coupled with glutathione–Sepharose-4B beads in PBS with Complete Protease Inhibition Cocktail tablets overnight, washed 5× with 0.25% Triton X-100 in PBS, boiled in SDS–PAGE sample buffer (25 mM

Tris–HCl, pH 6.8, 2.5% SDS, 2.5% glycerol, and 100mM DTT) for 5 min, resolved by SDS–PAGE, transferred to polyvinylidene difluoride membranes, probed with anti-β-adducin specific rabbit polyclonal antibodies (kindly provided by Dr.Vann Bennett, Duke University, Durham, NC), and incubated with anti-rabbit IgG HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using the chemiluminescence ECL Kit obtained from Amersham Biosciences, Piscataway, NJ.

Dephosphorylation of β-adducin phosphorylated in tyrosine by RPTPβ/ζ. Three 100-mm plates of HeLa cells were grown to 70% confluence in DMEM with 10% FBS, serum starved for 24 h, and stimulated with PTN 50 ng/ml (R&D Systems, Minneapolis, MN) for 30 min. The cells were lysed and samples were incubated with either GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S) for 2 h in 25 mM Tris–HCl, pH 7.2, 10 mM DTT in PBS at 37 °C. After 2 h of incubation, β-adducin was immunoprecipitated from the lysates with 6 μg anti-β-adducin antibodies coupled to 50 μl of protein G–Sepharose beads and incubated overnight at 4 °C. Protein G coupled β-adducin was washed with 1% Triton X-100 in PBS, eluted, and analyzed in Western blots probed with anti-phosphotyrosine antibodies (BD Transduction Laboratory, La Jolla, CA). The blots were stripped and re-probed with anti-β-adducin to confirm the identity of β-adducin.

Results

Yeast two-hybrid screen: identification of the 191 C-terminal amino acid fragment of β-adducin

To seek proteins potentially targeted by PTN through its interaction and consequent inactivation of RPTPβ/ζ, a human fetal brain cDNA library was screened with the intracellular domain of RPTPβ/ζ as "bait" in a yeast two-hybrid screen as described under Materials and methods above. Eighty-eight yeast colonies were identified that grew under the stringent nutrient selective conditions and 12 of these grew under the most nutrient selective conditions. The inserts in each of the 12 colonies were sequenced and the DNA sequence of one of the isolated clones encoded a in-frame, 191 amino acid sequence that is identical to the 191 amino acid C-terminal sequence of human β-adducin (residues 535–726) (Fig. 2). Plasmids encoding the RPTPβ/ζ cytoplasmic "bait" domain and the 191 C-terminal fragment of β-adducin were used to transform A109 yeast cells. The cells formed large colonies under the most stringent nutrient selective conditions and expressed high levels of the reporter gene β-galactosidase, confirming that β-adducin associates with the intracellular domain of RPTPβ/ζ in the yeast two-hybrid system.

Analysis of the predicted sequence of the 191 amino acid C-terminal fragment identified a single putative tyrosine phosphorylation site LEE(Y564)KK. This putative phosphorylation site in β-adducin is nearly identical in amino acid sequence to a putative tyrosine phosphorylation site at the identical locus in the C-terminal region of both α-adducin and γ-adducin, and to a remarkably similar site in all vertebrate adducins screened, including *Gallus gallus* γ-adducin and *Danio rerio* α-adducin as shown in Fig. 3, suggesting that this putative tyrosine phosphorylation site is of high importance and perhaps needed for adducin functions, suggesting also it is this site in β-adducin that is recognized by the intracellular domain of RPTPβ/ζ.

β-adducin interacts with the active site containing (D1) domain of RPTPβ/ζ

The possibility that β-adducin is recognized by the active site D1 domain of RPTPβ/ζ was first tested. Residues 1663–2034 of RPTPβ/ζ, which contain the active site D1 domain of RPTPβ/ζ, and residues 1663–2034 (C1932S) of RPTPβ/ζ, which contain the inactivated catalytic cysteine (1932) active site mutant D1 domain of RPTPβ/ζ, were fused at their C-termini to glutathione-*S*-transferase (GST), coupled to glutathione–Sepharose beads, and

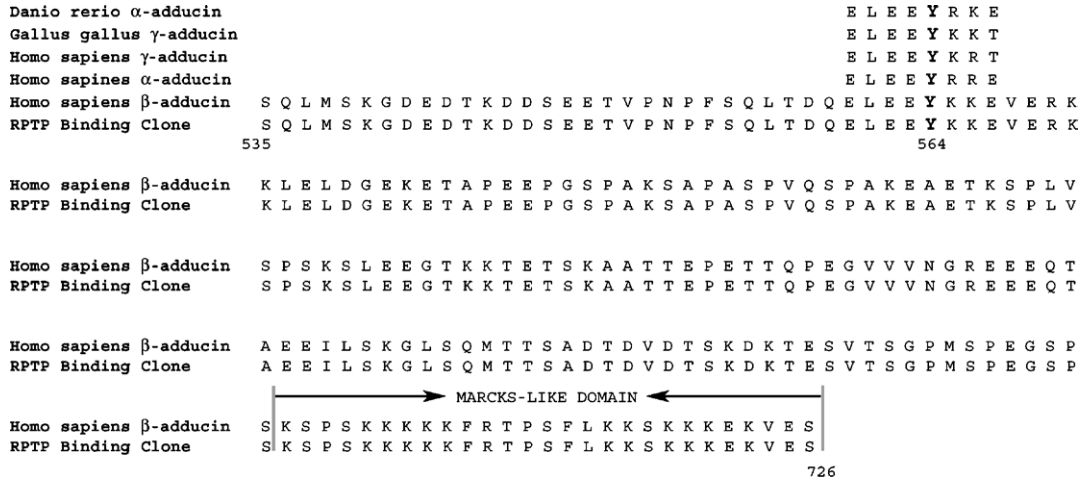

Fig. 3. Identification of the 191 C-terminal amino acid sequence of β-adducin. Amino acid sequence of the RPTPβ/ζ interactive isolated β-adducin clone (amino acid 535–726) and the α- and γ-adducin isoforms. Residue 564 is the putative tyrosine phosphorylation site in β-adducin and highly conserved in α- and γ-adducin as well as *D. rerio* and *G. gallus* adducins. The MARCKS domain in β-adducin (**KSPSKKKKKFRTPSFLKKSKKKEKVES**), (residue 700–726), derived from the similar motif in the MARCKS protein is highlighted.

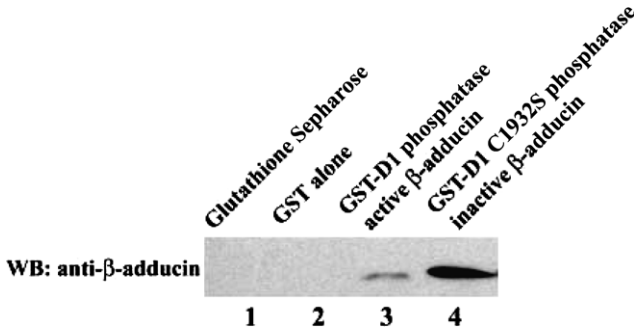

Fig. 4. RPTPβ/ζ D1 domain of capture. Lysates from HeLa cells were incubated with glutathione–Sepharose alone (lane 1), GST alone (lane 2), GST-D1 domain of RPTPβ/ζ (lane 3) and GST-D1 (C1932S) domain of RPTPβ/ζ (lane 4). The GST-coupled proteins "captured" from the lysates were probed with an anti-β-adducin antibody in Western blots.

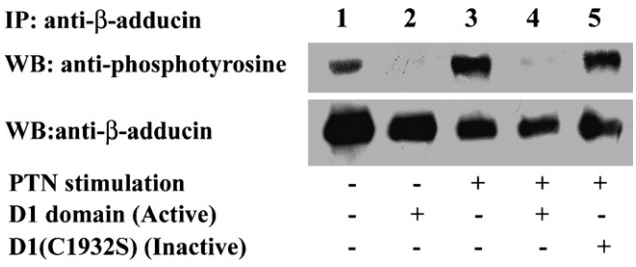

Fig. 5. Dephosphorylation of β-adducin from PTN-stimulated HeLa cells by the D1 domain of RPTPβ/ζ. Lysates from HeLa cells not stimulated (lane 1 and 2) and stimulated with PTN (lane 3–5) were immunoprecipitated with anti-β-adducin antibodies and incubated with the RPTPβ/ζ D1 phosphatase active (lanes 2 and 4) or RPTPβ/ζ D1 (C1932S) (lane 5) phosphatase inactive domain of RPTPβ/ζ and analyzed in Western blots probed with an anti-phosphotyrosine antibodies and re-probed with anti-β-adducin antibodies. Lane 1, β-adducin is phosphorylated in tyrosine in HeLa cells not stimulated with PTN, lane 2, β-adducin in HeLa cells not stimulated with PTN is dephosphorylated by RPTPβ/ζ D1, lane 3, The steady-state levels of tyrosine phosphorylation of β-adducin are increased in PTN-stimulated HeLa cells. Lane 4, β-adducin phosphorylated in tyrosine is dephosphorylated by the phosphatase activity of RPTPβ/ζ D1. Lane 5, β-adducin phosphorylated in tyrosine is not dephosphorylated by the inactivated RPTPβ/ζ D1 (C1932S).

incubated with lysates of confluent HeLa cells. Proteins "captured" by GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932S) were eluted with 1 mM glutathione and analyzed in Western blots probed with anti-β-adducin- specific antibodies. Both GST-RPTPβ/ζ D1 and GST-RPTPβ/ζ D1 (C1932S) captured a protein recognized in Western blots as β-adducin (Fig. 4, lanes 3 and 4) but GST alone coupled to glutathione–Sepharose (as control) did not, demonstrating that β-adducin interacts with the active site containing D1 domain of RPTPβ/ζ in GST-capture assays.

GST-RPTPβ/ζ D1 (C1932S) captured significantly more β-adducin from the same cell lysates than was captured by GST-RPTPβ/ζ D1 (Fig. 4, lanes 3 and 4), suggesting that in these lysates, β-adducin is phosphorylated in tyrosine and dephosphorylated during incubation by GST-RPTPβ/ζ D1. In this case, β-adducin dephosphorylated in tyrosine, the product of GST-RPTPβ/ζ D1, is anticipated to have a major loss of affinity for GST-RPTPβ/ζ D1, resulting in its capture significantly less efficiently by GST-RPTPβ/ζ D1 than by GST-RPTPβ/ζ D1 (C1932S).

This result thus not only demonstrated the association of β-adducin with the active site domain of RPTPβ/ζ, but supported the possibilities that β-adducin is phosphorylated in tyrosine in lysates of HeLa cells and a substrate of RPTPβ/ζ.

Pleiotrophin increases the steady-state levels of tyrosine phosphorylation of β-adducin in PTN-stimulated cells β-adducin is a substrate of RPTPβ/ζ

To test the possibility that β-adducin is a target of the PTN/RPTPβ/ζ signaling pathway, lysates were prepared from control HeLa cells (Fig. 5, lanes 1 and 2) and from HeLa cells that were stimulated with 50 ng/ml PTN for 30 min (Fig. 5, lanes 3–5). The lysates were immunoprecipitated with anti-β-adducin antibodies, and the immunoprecipitates were solubilized and analyzed in Western blots probed with anti-phosphotyrosine antibodies. To confirm the identity of the phosphoproteins identified with anti-phosphotyrosine antibodies as β-adducin, the blots were then stripped and reprobed with anti-β-adducin-specific antibodies. It was found that β-adducin is phosphorylated in tyrosine in non-PTN-stimulated HeLa cells (Fig. 5, lane 1) as suggested above and that the levels of tyrosine phosphorylation of β-adducin are sharply increased in PTN-stimulated HeLa cells (Fig. 5, lanes 3 and 5). When lysates prepared from both PTN-stimulated and non-PTN-stimulated HeLa cells were incubated with either GST-RPTPβ/ζ D1 or GST-RPTPβ/ζ D1 (C1932S), the levels of β-adducin phosphorylated in tyrosine in lysates from both PTN-stimulated and non-PTN-stimulated cells were markedly reduced when incubated with GST-RPTPβ/ζ D1 (Fig. 5, lanes 2 and 4) but were not reduced when incubated with the activate site inactivated GST-RPTPβ/ζ D1(C1932S) (Fig. 5, lane 5), demonstrating directly that PTN sharply increases tyrosine phosphorylation of β-adducin in PTN-stimulated cells, that β-adducin is phosphorylated in tyrosine in both PTN-stimulated and non-stimulated cells, and that β-adducin phosphorylated in tyrosine is a substrate of RPTPβ/ζ D1, but is not dephosphorylated by RPTPβ/ζ D1 (C1932S).

Discussion

The data presented here demonstrate that β-adducin is a substrate of RPTPβ/ζ and a downstream target of

the PTN/RPTPβ/ζ signaling pathway. The data demonstrate that PTN regulates β-adducin through its ability to regulate the steady-state levels of tyrosine phosphorylation of β-adducin and that exogenous PTN stimulates a marked increase in the levels of tyrosine phosphorylation of β-adducin. The data suggest that PTN regulates β-adducin and the previously identified RPTPβ/ζ substrate β-catenin in concert in PTN-stimulated cells and thus PTN-stimulated tyrosine phosphorylation of β-adducin may participate coordinately with β-catenin in the PTN-stimulated desestabilization of cytoskeleton, increased plasticity, and loss of homophilic cell–cell adhesion that is characteristic of PTN-stimulated cells. The data furthermore are consistent with the conclusion that the endogenous PTN/RPTPβ/ζ signaling pathway functions to maintain the equilibrium of phosphorylated and non-phosphorylated levels β-adducin and β-catenin in different cellular contexts and thus to "fine tune" cytoskeletal structure and fluidity through this mechanism.

β-adducin is known to be an important cytoskeletal protein; it forms hetero-tetramers with α-adducin which associate with the growing ends of actin filaments and with spectrin/actin junction complexes [45,48] and thus to stabilize actin–spectrin junctions near the cell membrane [43–47]. The adducin isoforms comprise N-terminal globular head domains (39 kDa) that are important in the formation of the αβ-adducin hetero-tetrameric complex, small neck domains (9 kDa), and protease sensitive tail domains that directly bind to both actin and spectrin [45,49]. The adducin isoforms share a C-terminal myristoylated alanine-rich protein kinase C substrate (MARCKS)-like domain that contains the important functional protein kinase C (PKC) phosphorylation sites at serines 713 and 726. When serines 713 and 726 are phosphorylated, the affinity of β-adducin with actin and spectrin is sharply reduced, leading to disruption of the spectrin/actin/adducin membrane-associated network, cytoskeletal remodeling, and increased cytoskeletal plasticity.

A recent report [50] demonstrated that β-adducin transiently co-expressed together with the Src family member Fyn is phosphorylated and recruited to the plasma membrane. It was suggested that tyrosine phosphorylation of β-adducin decreases the pool of available β-adducin necessary to directly stabilize the actin cytoskeleton, thereby permitting cells to change shape or to initiate migration. These results [50] thus support the hypothesis that the PTN-stimulated increase in tyrosine phosphorylation of β-adducin is important and it is therefore possible that PTN-stimulated tyrosine phosphorylation of β-adducin may contribute to desestabilization of cytoskeletal complexes and cytoskeletal plasticity through the initiation of migration of β-adducin in PTN-stimulated cells. These findings also raise the possibility that Fyn is the tyrosine kinase whose activity increases the levels of tyrosine phosphorylation of β-adducin in PTN-stimulated cells.

Recently, GIT1/Cat-1 also was identified as a substrate of RPTPβ/ζ using a yeast substrate trapping system [51], expanding the tyrosine phosphorylated proteins that are substrates of RPTPβ/ζ and thus downstream targets of the PTN/RPTPβ/ζ signaling pathway. Overexpression of GIT1 in HEK293 cells inhibits G protein-coupled receptor internalization [52,53] and tyrosine phosphorylation of GIT1 is increased following cell spreading on fibronectin. The levels of tyrosine phosphorylation of GIT1 are regulated during the cell cycle [54], potentially also expanding the cellular regulatory systems regulated by PTN. When we searched different data bases, similarities in potential phosphorylation sites in GIT1/Cat-1 with β-catenin and β-adducin were not found, supporting the hypothesis that RPTPβ/ζ substrate specificity is promiscuous. The promiscuity of substrate specificity suggests the likelihood that a hierarchy of many RPTPβ/ζ substrate proteins is regulated by tyrosine phosphorylation through the PTN/RPTPβ/ζ signaling cascade, and the degree of their regulation is dependent on their relative affinity for RPTPβ/ζ and their relative abundance.

In summary, this work has uncovered β-adducin as a novel downstream target in the PTN-signaling pathway. It was found that PTN regulates the steady-state levels of tyrosine phosphorylation of β-adducin and, suggested that, through regulation of tyrosine phosphorylation levels of β-adducin, PTN may coordinate the functional activities of β-adducin with β-catenin to regulate cytoskeletal remodeling.

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Pleiotrophin regulates serine phosphorylation and the cellular distribution of β-adducin through activation of protein kinase C

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Pleiotrophin (PTN) was found to regulate tyrosine phosphorylation of β-adducin through the PTN/receptor protein tyrosine phosphatase (RPTP)β/ζ signaling pathway. We now demonstrate that PTN stimulates the phosphorylation of serines 713 and 726 in the myristoylated alanine-rich protein kinase (PK) C substrate domain of β-adducin through activation of either PKC α or β. We also demonstrate that PTN stimulates translocation of phosphoserine 713 and 726 β-adducin either to nuclei, where it associates with nuclear chromatin and with centrioles of dividing cells, or to a membrane-associated site, depending on the phase of cell growth. Furthermore, we demonstrate that PTN stimulates the degradation of β-adducin in PTN-stimulated cells. Phosphorylation of serines 713 and 726 in β-adducin is known to markedly reduce the affinity of β-adducin for spectrin and actin and to uncouple actin/spectrin/β-adducin multimeric complexes needed for cytoskeletal stability. The data thus suggest that the PTN-stimulated phosphorylation of serines 713 and 726 in β-adducin disrupts cytoskeletal protein complexes and integrity, features demonstrated in both PTN-stimulated cells and of highly malignant cells that constitutively express the endogenous *Ptn* gene. The data also support the important conclusion that PTN determines the cellular location of β-adducin phosphorylated in serines 713 and 726 and raise the possibility that β-adducin functions in support of structure of heterochromatin and centrioles during mitosis.

Pleiotrophin (PTN the protein, *Ptn* the gene) is a secreted, highly conserved heparin-binding cytokine of 136 aa (1–3). Pleiotrophin is >50% identical in amino acid sequence and shares striking structural and functional similarities with midkine (MK the protein, *Mk* the gene). Together, PTN and MK constitute the only members of the *Ptn/Mk* developmental gene family (1, 3–5). Pleiotrophin stimulates proliferation of cultured fibroblasts (1, 3, 5), endothelial cells (6–9), epithelial cells (refs. 7 and 10–12, and N. Zhang, R. Zhong, Z. Y. Wang, and T.F.D., unpublished data). The product of the endogenous *Ptn* gene initiates the spontaneous lineage-specific differentiation that is characteristic of oligodendrocyte progenitors in primary culture through an autocrine mechanism (ref. 9 and H. J. Yeh, I. Silos-Santiago, M. Gurrieri, S. Jhaveri, Y. S. Li, and T.F.D., unpublished data).

The *Ptn* gene also is a proto-oncogene (12, 13). Its high-level importance in human malignancies is suggested because expression of the endogenous *Ptn* gene is seen frequently in many different highly malignant human neoplasms (14–22), and introduction of a dominant negative *Ptn* gene or targeted ribozymes into cells from malignancies with constitutive expression of the *Ptn* gene reverses the malignant phenotype to the phenotype of the premalignant cells both *in vivo* and *in vitro*, indicating that PTN signaling is responsible for the "switch" of the premalignant cell to the highly malignant phenotype (11, 12, 21, 23, 24).

Recent studies have uncovered clues to the mechanisms by which PTN initiates this striking diversity of phenotypes in PTN-stimulated cells. Pleiotrophin signals through two functionally and structurally independent signaling domains (refs. 5, 11, and 12 and N. Zhang, R. Zhong, Z. Y. Wang, and T.F.D., unpublished data), each of which binds heparin (25), and each of which signals very different phenotypes. The N-terminal domain, when expressed with the endogenous signal peptide, transforms murine fibroblasts, whereas the C-terminal domain does not; however, the C-terminal domain of PTN induces rapid growth and a striking angiogenic phenotype when it is expressed with the endogenous signal peptide in the premalignant cell. Thus, the separate domains of PTN function independently of each other, signal different phenotypes, and, *a priori*, need to signal through two separate receptor-like proteins (5, 11, 12).

One receptor that initiates PTN signaling is the transmembrane receptor protein tyrosine phosphatase (RPTP) β/ζ. The interaction of PTN with RPTPβ/ζ induces receptor dimerization and inactivates the endogenous tyrosine phosphatase activity of RPTPβ/ζ, thereby disrupting the balanced activity of RPTPβ/ζ and an unknown but constitutively active tyrosine kinase(s) on the mutual substrates of RPTPβ/ζ and the tyrosine kinase and thus initiating a sharp and rapid increase in the steady state levels of tyrosine phosphorylation (26). The first substrate of RPTPβ/ζ to be identified was β-catenin (26) and, more recently, β-adducin also was found to be a substrate of RPTPβ/ζ (47), thereby focusing attention on the cytoskeletal proteins as one target of the PTN/RPTPβ/ζ signaling pathway. In the case of β-catenin, the PTN-stimulated increase in tyrosine phosphorylation has led to the disruption of the association of β-catenin with the cadherin family, loss of adherins junction protein complexes, and loss of cell–cell adhesion (P.P.-P., Y. Chang, J. A. Vega, and T.F.D., unpublished data). β-Adducin was found to be interactive with the intercellular domain of the PTN receptor RPTPβ/ζ in a yeast two-hybrid system (47) and found to be a downstream target of the PTN/RPTPβ/ζ signaling pathway, suggesting that, together with β-catenin, β-adducin may have a role in the cytoskeletal rearrangements and fluidity characteristic of cells stimulated by PTN (unpublished data).

Here, we demonstrate that PTN activates protein kinase C (PKC) activity in PTN-stimulated cells, initiates phosphorylation of serines 713 and 726 in β-adducin through the PTN-dependent activated PKC activity, and redistributes β-adducin phosphorylated in serines 713 and 726 to different cellular sites, as well as targeting β-adducin for proteolysis. Because phosphorylation of serines 713 and 726 is known to markedly reduce the affinity of β-adducin for actin and spectrin, the data support the conclusion that PTN both stimulates cytoskeletal instability and plasticity through serine phosphorylation of β-adducin and redistributes serine phosphorylation β-adducin to determine its activity at different cellular sites. Furthermore, it is shown that β-adducin associates with heterochromatin and centrioles, raising the impor-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; MARCKS, myristoylated alanine-rich PKC substrate; RPTP, receptor protein tyrosine phosphatase.

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tant possibility that β-adducin functions to stabilize these structures in addition to its well known functions in regulating the stability of the cell membrane associated cytoskeleton.

Materials and Methods

Cell Culture. HeLa (American Type Culture Collection) cells were used in all experiments and were cultured in DMEM with 10% FBS unless otherwise noted.

Stimulation of HeLa Cells with Different Reagents in Culture. HeLa cells were grown to 70% confluence in 100-mm plates, serum starved for 24 h, and treated with 50 ng/ml PTN (R & D Systems) for different times, 0.1 μM phorbol 12-myristate 13-acetate (PMA) for 12 h, or 10 μM of a PKC α/β pseudosubstrate inhibitor of PKC (Calbiochem) for 1 h as indicated.

Western Blot Analysis. Lysates of cells were prepared in PBS (pH 7.2) with 1% SDS, 1 mM PMSF, 10 mg/ml leupeptin, and 1× Complete Protease Inhibition tablets (Roche Diagnostics), resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti-β-adducin antibodies (kindly provided by Vann Bennet, Duke University, Durham, NC) and mouse anti-phosphoserine 713 and 726 β-adducin-specific antibodies (Upstate, Charlottesville, VA) at a 1:1,000 dilution, mouse anti-phosphoadducin antibodies that react with phosphoserine 713 β-adducin and phosphoserine 724 α-adducin (Upstate) at a 1:1,000 dilution, and rabbit anti-phosphoserine 152 and 156 myristoylated alanine-rich PKC substrate (MARCKS)-specific antibodies (Upstate) at a 1:1,000 dilution, or mouse anti-actin antibodies at a 1:2,500 dilution as indicated. Immunoreactive proteins were detected with either goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology), and illuminated with the ECL detection system (Amersham Pharmacia).

Immunocytochemistry. HeLa cells were seeded on coverslips at either 0.5×10^6 or 9×10^6 cells per ml, as indicated, and cultured for 24 h. The cells were then serum starved for 24 h, treated with 50 ng/ml PTN for 60 min, washed twice in ice cold PBS (pH 7.2), and fixed for 30 min with 4% paraformaldehyde in PBS. The levels of nonspecific protein binding were reduced with 1% FBS in PBS with 0.1% Triton X-100 for 2 h, incubated with a 1:200 dilution of mouse anti-phosphoserine 713 and 726 β-adducin-specific antibodies (Upstate) overnight in PBS with 0.1% Triton X-100, washed three times for 10 min in PBS, and treated with goat anti-mouse FITC conjugated antibodies (diluted 1:1,000; Chemicon). Actin and nuclei were stained with 1 μM phalloidin and DAPI (1:15,000) (Sigma), respectively, for 10 min in 0.05% Triton X-100 in PBS, washed four times for 10 min in PBS, mounted by using ProLong Antifade kit (Molecular Probes) on slides, and visualized by confocal microscopy in a Nikon Eclipse E600 microscope.

PKC Activity Assay. PKC activity was measured by using a PKC Kinase Non-Radioactive Assay kit (Stressgen, Victoria, Canada) as follows: HeLa cells were grown to confluency in six-well plates, serum starved for 24 h, and treated in triplicate with 1 μM PMA for 1 h or with 50 ng/ml PTN for 25 min, washed twice with PBS at 4°C, and lysed in sample preparation lysis buffer (20 mM Mops, pH 7.2/1 mM sodium vanadate/5 mM EGTA/2 mM EDTA/1% Nonidet P-40/1 μM DTT/1 mM PMSF/10 μg/ml leupeptin and aprotinin in PBS) and centrifuged at $15,000 \times g$ for 15 min. The supernatant protein concentration was determined with the standard Lowry assay. Eighteen micrograms of lysate protein was diluted into 30 μl of the kinase assay dilution buffer (Stressgen) and loaded on 96-well plates coated with a PKC substrate peptide. The PKC assay was initiated by the addition of 10 μl of ATP (diluted 1 mg/ml) to each well at 30°C and assayed as per the manufacturer's instructions, measuring incorporation of phosphate into the substrate peptide at 50 min. The wells were then washed twice with antibody dilution buffer (Stressgen), and 40 μl of phosphospecific substrate antibodies were added to each well and incubated for 1 h. Each well was subsequently washed three times for 10 min with wash buffer (Stressgen) and a 1:1,000 dilution of anti-rabbit IgG HRP-conjugated antibody preparation in dilution buffer and incubated for 30 min. The wells were washed three times, and 60 μl of tetramethylbenzidine substrate (Stressgen) was added and incubated in the wells for 45 min. The HRP reaction was quenched with the addition of 20 μl of acid stop solution (Stressgen), and absorbance at 450 nm of each well was measured. The reaction was found to be linear with protein concentration and time.

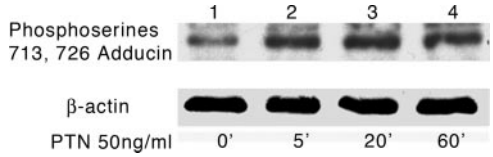

Fig. 1. Western blot of lysates from PTN-stimulated HeLa cells probed with anti-phosphoserine 713 and 726 β-adducin antibodies. (*Upper*) Lanes 1, untreated; 2, 5 min after PTN treatment; 3, 20 min after PTN treatment; 4, 60 min after PTN treatment. (*Lower*) Reprobe of Western blot with anti-actin antibodies.

Results

Pleiotrophin Stimulates Increased Phosphorylation of Serines 713 and 726 in β-Adducin. We found that the C-terminal 191-aa fragment of β-adducin interacts with the intracellular domain of RPTPβ/ζ in a yeast two-hybrid system (47). Furthermore, it was found that β-adducin interacts with the active site containing (D1) domain of RPTPβ/ζ, that β-adducin is a substrate of RPTPβ/ζ, and that PTN stimulates sharp increases in tyrosine phosphorylation of β-adducin in PTN-stimulated cells, presumably through phosphorylation of a highly conserved tyrosine phosphorylation site at Y564 found also in adducins α and γ (Fig. 7, which is published as supporting information on the PNAS web site). The C-terminal 191 aa fragment of β-adducin contains important phosphorylation sites at serines 713 and 726 within the MARCKS domain of β-adducin, which also are highly conserved in adducins α and γ and in the MARCKS protein itself (see Figs. 7 and 8, which are published as supporting information on the PNAS web site) (27). When serines 713 and 726 in β-adducin are phosphorylated, the affinity of β-adducin with actin and spectrin is reduced manyfold (27). Phosphorylation of serines 713 and 726 in β-adducin thus destabilizes the spectrin/actin/β-adducin protein complexes and consequently also destabilizes the spectrin membrane associated network and its association with filamentous actin needed for cytoskeletal stability. The steady state levels of serines 713 and 726 phosphorylation thus are critical determinants of the relative plasticity of cytoskeletal structures.

Because phosphorylation of serines 713 and 726 in β-adducin is required for the known disruption of cytoskeletal complexes and loss of homophilic cell–cell adhesion, which is a characteristic of cells stimulated by PTN, to seek a connection between these two phenomena, we treated HeLa cells with 50 ng/ml PTN for different times, and the levels of phosphoserines 713 and 726 in β-adducin were measured in Western blots of lysates of PTN-treated and control, nontreated, cells probed with anti-phosphoserine 713 and 726 β-adducin antibodies. Fig. 1 demonstrates that the levels of phosphorylation of serines 713 and 726 β-adducin increase progressively with time of exposure to

PTN in PTN-stimulated cells; a significant increase in phosphorylation of serines 713 and 726 β-adducin was seen within 5 min of stimulation (Fig. 1, lane 2); a further increase in the phosphorylation was seen at 20 min (Fig. 1, lane 3), and the levels then remained essentially constant to 1 h (Fig. 1, lane 4). Pleiotrophin thus stimulates a rapid, time-dependent, and readily detectable increase in the levels of phosphoserine 713 and 726 β-adducin in HeLa cells. In contrast, the levels of phosphoserine 713 and 726 β-adducin in the cells not stimulated with PTN remained constant for the 60-min period of incubation (data not shown).

Interestingly, HeLa cells that were not stimulated by PTN also have readily detectable levels of phosphoserine 713 and 726 β-adducin when lysates were analyzed in Western blots (Fig. 1, lane 1), suggesting that, in nonstimulated cells, an equilibrium of phosphorylated and nonphosphorylated serines 713 and 726 β-adducin may determine the relative cytoskeletal fluidity, and that the endogenous PTN/RPTPβ/ζ signaling pathway may be a central regulator of the fluidity of the cytoskeleton in cells that have not been stimulated with exogenous PTN.

Pleiotrophin Stimulates Redistribution of Phosphoserine 713 and 726 β-Adducin to Different Cellular Sites. We also examined PTN-stimulated cells stained with fluorescent-tagged anti-phosphoserine 713 and 726 β-adducin antibodies with confocal microscopy. In a control study to confirm an optimal time for cells to be examined after stimulation with PTN, HeLa cells in log phase growth and at confluence were first stimulated with 50 ng/ml PTN for 60 min. Cells both in log phase growth and at confluence stimulated with PTN were found to have readily detected increases of phosphorylation of serines 713 and 726 in β-adducin in lysates when examined in Western blots probed with anti-phosphoserine 713 and 726 antibodies (data not shown). When non-PTN-stimulated, nonconfluent HeLa cells were treated with anti-phosphoserine 713 and 726 β-adducin antibodies and examined by confocal microscopy, the fluorescent signal generated by the anti-phosphoserine 713 and 726 β-adducin antibodies was nearly exclusively limited to nuclei (Fig. 2*B*). However, when these cells were stimulated with PTN, a sharp increase in the levels of the fluorescent signal generated by the anti-phosphoserine 713 and 726 β-adducin antibodies was found in the cytosol (Fig. 2*D*, compared to *B*, arrows); the increase in the cytoplasmic signal in PTN-stimulated nonconfluent cells stained with anti-phosphoserine 713 and 726 β-adducin antibodies, DAPI, and phalloidin is seen more clearly (Fig. 2*C*, compared to *A*, arrows). The β-adducin phosphorylated in serines 713 and 726 is diffusely spread throughout the cytosol and appears to be localized in small endocytic vesicles (Fig. 2 *C* and *D*), suggesting that PTN may signal the degradation of β-adducin in PTN-stimulated nonconfluent HeLa cells as well as its redistribution. The increase in serine 713 and 726 β-adducin in PTN-stimulated cells in cytosol is presumed to be derived by translocation from nucleus; however, the density of the nuclear signal was sufficiently high that it was not possible to correlate a loss of the nuclear signal with the increase in cytosolic fluorescent staining that was observed.

When confluent, non-PTN-stimulated HeLa cells were examined by confocal microscopy, the fluorescent signal generated with anti-phosphoserine 713 and 726 antibodies was distinctly localized to regions of cell–cell contact (Fig. 2*F*); however, when these cells were stimulated with PTN, a striking increase in the fluorescent signal generated by anti-phosphoserine 713 and 726 β-adducin antibodies was found in nuclei, accompanied by a sharp loss of the fluorescent signal from regions of cell–cell contact. The fluorescent signal in the PTN-stimulated confluent cells was found diffusely spread in cytoplasm (Fig. 2*H*). Importantly, when PTN-stimulated cells were stained with anti-phosphoserine 713 and 726 β-adducin, DAPI, and phalloidin, phosphoserine 713 and 726 β-adducin was seen to colocalize with nucleus but also was found diffusely spread in cytoplasm (Fig. 2*G*).

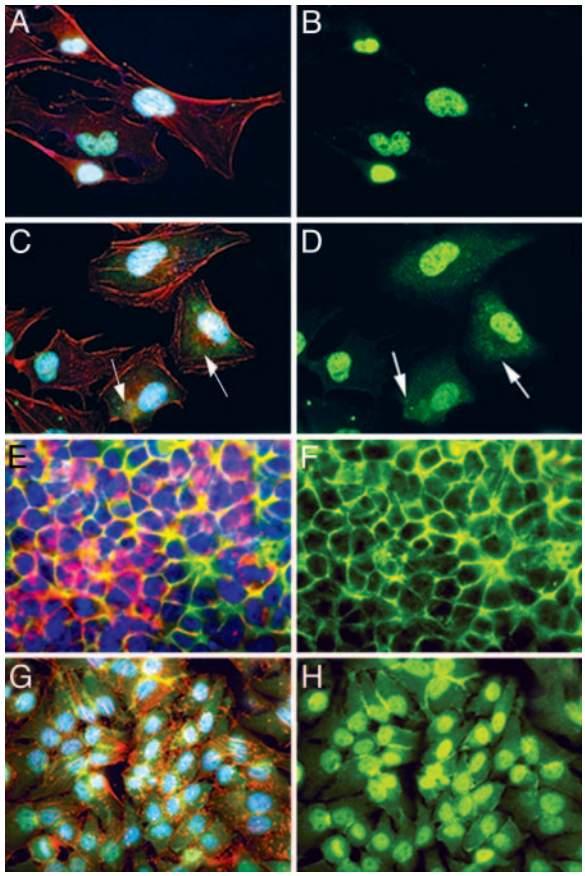

Fig. 2. Cellular localization of β-adducin in confluent and nonconfluent non-PTN-treated (control) and PTN-treated HeLa cells. (*A*) Non-PTN-treated sparse HeLa cells stained with phalloidin (red), anti-phosphoserine 713 and 726 β-adducin antibodies (green), and DAPI (blue). (*B*) Non-PTN-treated sparse HeLa cells stained with anti-phosphoserine 713 and 726 β-adducin antibodies. (*C*) PTN-treated (50 ng/ml) sparse HeLa cells for 60 min stained with phalloidin, anti-phosphoserine 713 and 726 β-adducin antibodies, and DAPI. (*D*) PTN-treated (50 ng/ml) sparse HeLa cells for 60 min stained with anti-phosphoserine 713 and 726 β-adducin antibodies. (*E*) Non-PTN-treated confluent HeLa cells stained with phalloidin, anti-phosphoserine 713 and 726 β-adducin antibodies, and DAPI. (*F*) Non-PTN-treated confluent HeLa cells stained with anti-phosphoserine 713 and 726 β-adducin antibodies. (*G*) PTN-treated (50 ng/ml) confluent HeLa cells for 60 min stained with phalloidin, anti-phosphoserine 713 and 726 β-adducin antibodies, and DAPI. (*H*) PTN-treated (50 ng/ml) confluent HeLa cells for 60 min stained with anti-phosphoserine 713 and 726 β-adducin antibodies.

Rapid Loss of β-Adducin Protein in PTN-Stimulated Cells. Because of the apparent degradation of β-adducin in log phase growing HeLa cells stimulated with PTN, lysates of HeLa cells in log phase growth stimulated with PTN at different times were analyzed in Western blots probed with anti-β-adducin antibodies. In Fig. 3, a time-dependent loss of β-adducin in nonconfluent HeLa cells is demonstrated, whereas no loss of the levels of the actin control were found, supporting strongly the view that PTN not only redistributes phosphoserine 713 and 726 β-adducin in both log phase and confluent HeLa cells, but also signals a rapid loss of β-adducin protein.

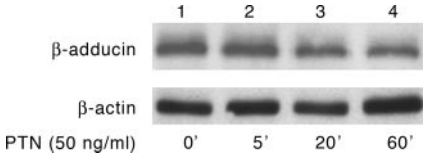

Fig. 3. Western blot of lysates of the time-dependent PTN-stimulated nonconfluent HeLa cells probed with anti-β-adducin antibodies.

β-Adducin Phosphorylated at Serines 713 and 726 Is Associated with Nuclear Chromatin and Centrioles. The nuclear fluorescent staining generated by anti-phosphoserine 713 and 726 β-adducin antibodies in confocal microscopy was surprising, because only a single reference of β-adducin associated with nuclei in mammalian cells was found in searches of different databases (28); in that study, immunoreactive β-adducin was seen in nuclei of mouse oocytes; it was suggested that β-adducin was associated with nuclear chromatin. Phosphoserine 713 and 726 β-adducin in nuclei in our studies appeared also to associate with nuclear chromatin (Fig. 2*G*). To confirm this impression, images of cells

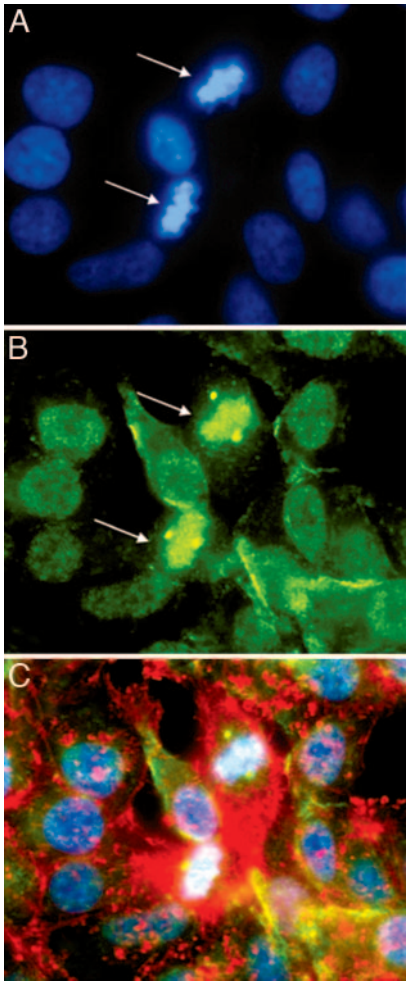

Fig. 4. Localization of β-adducin in centrioles of PTN-treated dividing HeLa cells. (*A*) PTN-treated (50 ng/ml) HeLa cells stained with DAPI (blue). (*B*) PTN-treated (50 ng/ml) HeLa cells stained with anti-phosphoserine 713 and 726 β-adducin antibodies (green). (*C*) PTN-treated (50 ng/ml) HeLa cells stained with phalloidin (red), anti-phosphoserine 713 and 726 β-adducin antibodies (green), and DAPI (blue). Note the localization of phosphoserine 713 and 726 β-adducin to chromatin and the centrioles during metaphase (arrows).

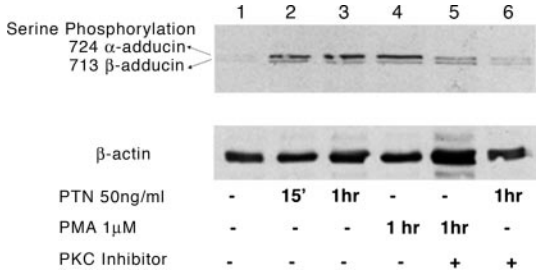

Fig. 5. Both PTN and PMA enhance phosphorylation of β-adducin residue 713 and α-adducin residue 724. (*Upper*) Western blot of lysates from PTN-treated (50 ng/ml, for 15 and 60 min), PMA-tretaed (1 μM, 60 min), or PKCα/β inhibitor-treated (60 min before PTN and PMA treatment) HeLa cells probed with anti-phosphoserine 713 β-adducin and anti-phosphoserine 724 α-adducin antibodies. (*Lower*) Reprobe of Western blot with an anti-actin-specific antibody.

were again stained with DAPI (Fig. 4*A*) and anti-phosphoserine 713 and 726 β-adducin (Fig. 4*B*) and superimposed with images of the same cells (Fig. 4*C*). We found that phosphoserine 713 and 726 β-adducin associates directly with nuclear chromatin in the superimposed image.

Remarkably, the fluorescent stain representing phosphoserine 713 and 726 β-adducin was prominently localized in centrioles as well as nuclear chromatin (Fig. 4, arrows denote centrioles). To the best of our knowledge, the identification of phosphoserine 713 and 726 β-adducin in centrioles in mammalian cells has not been described, raising the distinct possibility that β-adducin may stabilize the centriole protein complexes during mitosis.

Pleiotrophin Stimulates Activation of PKC. Different studies demonstrate that phosphorylation of serine 713 and 726 β-adducin is catalyzed by a PKC (27). Lysates from the PMA-stimulated and PTN-stimulated confluent HeLa cells were therefore analyzed in Western blots probed with a different antibody preparation from that used in Fig. 1. This antibody (see *Materials and Methods* above) reacts with phosphoserine 713 of β-adducin and 724 of α-adducin, and was used to confirm the results obtained in Fig. 1 and, furthermore, to determine whether α-adducin is also phosphorylated in PTN-stimulated cells. It was found that PMA increased the phosphorylation of serine 713 β-adducin (Fig. 5 *Upper*, lower band) and 724 in α-adducin (Fig. 5 *Upper*, upper band) in lysates of PMA-stimulated cells (Fig. 5, lane 4) to levels similar to those found in lysates of PTN-stimulated cells (Fig. 5, lane 3). The PMA- and PTN-stimulated increases in levels of phosphoserines 713 in β-adducin and 724 α-adducin were markedly reduced when the pseudosubstrate peptide inhibitor of PKC α/β was included in cultures stimulated with either PMA (Fig. 5, lane 5) or PTN (Fig. 5, lane 6), supporting the conclusions that PTN and, likely, PMA activate either PKC α or β (or both) to catalyze the PMA- and PTN-stimulated increase in phosphoserine 713 in β-adducin and 724 in α-adducin.

We also tested the ability of PTN to stimulate serines 152 and 156 of a second important cytoskeleton protein and known PKC substrate, the MARCKS protein (29). The MARCKS protein contains the important regulatory "MARCKS" domain homologous with the MARCKS domain in β-adducin. Lysates from HeLa cells stimulated with 50 ng/ml PTN and PMA for 30 and 60 min were prepared and analyzed in Western blots probed with anti-phosphoserine 152 and 156 MARCKS antibodies. Both PMA and PTN stimulated a marked increase in phosphorylation of serine 152 and 156 MARCKS. The increase in phosphorylation of serine 152 and 156 MARCKS is both PTN concentration- and time-dependent to 60 min. Levels of phosphoserine 152 and

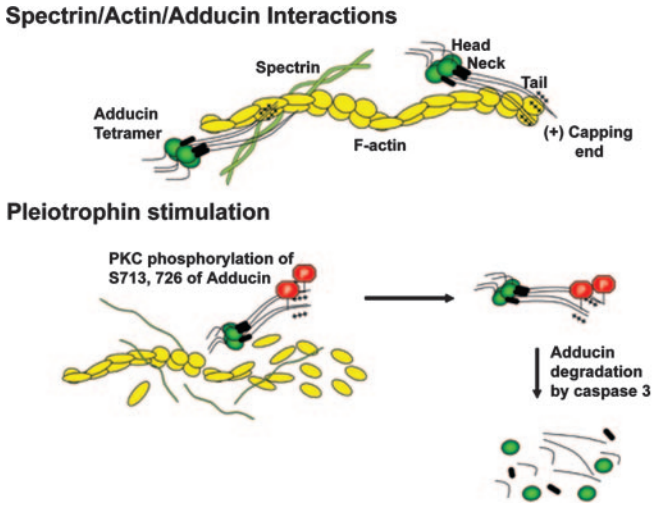

Fig. 6. Proposed model for PTN-stimulated dissociation of β-adducin from F-actin and spectrin. Modified from Bennet and Bainnes (32). β-Adducin forms complexes between spectrin and actin promoting the association of spectrin with actin filaments. Pleiotrophin-induced phosphorylation of serines 713 and 726 β-adducin reduces the affinity of β-adducin for filamentous actin dissociating these cytoskeletal structures and increasing cleavage of β-adducin by caspase-3 and degradation of β-adducin.

156 MARCKS remained constant in cells that were not stimulated with PTN throughout the experiment (Fig. 9, which is published as supporting information on the PNAS web site).

The levels of PKC(s) activity in non-PTN-stimulated (control) and in PTN- and PMA-stimulated confluent HeLa cells were also directly measured (see *Materials and Methods* above). The PKC activity in lysates from PTN-stimulated cells lysates was ≈42% above the endogenous activity in non-PMA-stimulated cells, and that in lysates from PMA-stimulated cells was ≈62% above the endogenous activity in non-PMA-stimulated cells (Fig. 10, which is published as supporting information on the PNAS web site). The data make clear that the baseline PKC activity of HeLa cells is high, a finding that is consistent with the high baseline levels of phosphorylation of serines 713 and 726 in β-adducin both in logarithmic and confluent non-PTN-stimulated HeLa cells. The data also make clear that PTN activates one or more isoforms of PKC; however, the level of the PTN-dependent increase of PKC activity is masked because of high endogenous PKC activity in nonstimulated cells. The data thus suggest that the endogenous PKC activity functions to maintain the steady-state levels of serine 713 and 726 phosphorylation and point to the possibility that the endogenous PTN/RPTPβ/ζ pathway may regulate the stability of β-adducin, spectrin, and actin complexes and that this pathway is a principle regulator of MARCKS domain containing cytoskeletal proteins.

Discussion

β-Adducin is an important cytoskeletal protein; it belongs to a family of proteins that preferentially bind to actin–spectrin junctions (30–32) and functions to stabilize the growing actin filaments and actin–spectrin networks (33, 34) through the formation of complexes between spectrin and actin and the promotion of the association of spectrin with actin filaments (32) (Fig. 6). The affinity of β-adducin for this complex is greatest at the fast growing ends of actin filaments, where β-adducin contributes to actin filament stability. Adducin α and γ isoforms interact together with β-adducin to create heterotetrameric complexes at the growing ends of actin filaments and actin–spectrin junctions (32). Each isoform of adducin is comprised of an N-terminal globular head domain (39 kDa) that allows for tetramerization, a small neck domain (9 kDa), and the protease-sensitive tail domain containing a MARCKS like domain (32) that binds to actin and spectrin and thus contributes to the stability of adducin/actin/spectrin junctions as well as growing actin filaments. β-adducin is thus critically placed in the cytoskeleton in such a position that its regulation is required for it to function effectively in membrane stability and fluidity at different phases of the cell cycle.

The present experiments demonstrate that PTN stimulates phosphorylation of serines 713 and 726 in the MARCKS domain of β-adducin (and serine 724 in α-adducin) and serines 152 and 156 in the MARCKS protein itself through the activation of either PKC α or β and perhaps other PKC(s) isoforms. These studies also demonstrate that PTN stimulates the translocation of phosphoserine 713 and 726 β-adducin from a nuclear localization to cytosol in nonconfluent HeLa cells and from a plasma membrane-associated site to nuclei in PTN-stimulated confluent HeLa cells. Furthermore, it is shown that PTN stimulates the degradation of β-adducin itself. Finally, these studies have uncovered the presence of β-adducin in nuclei, associated with heterochromatin and, in dividing cells, in centrioles.

These findings may be very important, because the characteristic features of PTN-stimulated cells and the malignant cells that have acquired an activated endogenous *Ptn* gene during tumor progression include disruption of cytoskeletal protein complexes and cytoskeletal stability. These features fit well with the known property of phosphorylation of serines 713 and 726 in β-adducin to greatly reduce the affinity of β-adducin to filamentous actin, the actin–spectrin junctions, and the fast growing ends of actin, resulting in dissociation of these cytoskeletal structures, loss of integrity of actin–spectrin complexes (32), and the cleavage of β-adducin by caspase-3 and degradation of β-adducin through the proteosome degradation pathway (36) (Fig. 6). Thus, PTN stimulation may not only decrease the association of actin, spectrin, β-adducin complexes, but also reduce the cellular pool of β-adducin, which may additionally add to loss of actin cytoskeletal integrity in PTN-stimulated cells. In support of our findings, the ability of β-adducin to contribute to cytoskeletal integrity is known to be regulated by PKCs (27), and PKC α, β, and γ are known to phosphorylate the consensus RTPS sequences (serines 713 and 726) sites within the highly conserved 22-aa residues at the extreme C-terminal MARCKS domain of β-adducin (and adducin α and γ) (27).

In other studies, we demonstrated that PTN stimulates tyrosine phosphorylation of β-catenin in PTN-stimulated cells, and, as a consequence, the affinity of β-catenin with the intracellular domains of E-cadherin and filamentous actin is reduced and adherens junctions complexes are dissolved, leading to disruption of cytoskeletal integrity and loss of homophilic cell–cell adhesion (ref. 26 and P.P.-P., Y. Chang, J. A. Vega, and T.F.D., unpublished data). β-Catenin links the catenin family and the highly conserved cytoplasmic domain of cadherins with the actin cytoskeleton (37–41), and it is known that levels of tyrosine phosphorylation of β-catenin correlate directly with increased perturbation of the link of the cadherins and actin filaments and with decreased cell–cell adhesion (37, 42–44). The previous findings that PTN regulates tyrosine phosphorylation of β-catenin, coupled with the striking modifications in β-adducin in PTN-stimulated cells, fit well with the loss of cytoskeletal structure and cell–cell adhesion and the increases in membrane plasticity that are characteristic of PTN-stimulated cells. These studies also fit well with similar features that are characteristic of the different human malignant cells with mutations that initiate constitutive activation of the endogenous *Ptn* gene.

It is also likely to be important that PTN translocates phosphoserine 713 and 726 β-adducin to different cellular sites, depending on whether cells are confluent; thus, PTN regulates

the sites at which phosphoserine 713 and 726 β-adducin is located and where β-adducin protein complexes are less stable. Previous studies (45) demonstrated that, when Fyn and β-adducin are cotransfected, β-adducin was phosphorylated in tyrosine and translocated to the cell membrane. Whether the PTN-dependent increase in tyrosine phosphorylation is the signal to initiate this translocation is not clear; however, we have found that PTN increases tyrosine phosphorylation of Fyn (48), and this finding may link the studies of Shima *et al.* (45) with the studies presented here.

In these studies, β-adducin was also found in nucleus, presumably in association with nuclear chromatin and with centrioles in cells during cell division. Only a single report identifying β-adducin (through immunoreactivity with anti-β-adducin antibodies) in nuclei of cells in mammalian species was found (28). The C-terminal region of β-adducin shares sequence homology with other nuclear localization signals, supporting the view that the nuclear localization of phosphoserine 713 and 726 β-adducin is not artifactual. A C-terminal nuclear bipartite nuclear targeting consensus sequence (KKKKKFRTPSFLKKSK) has been identified at the C-terminal domain of β-adducin that may direct β-adducin to nuclei. We speculate that the translocation of phosphoserine β-adducin in response to PTN possibly results from phosphorylation of serines 713 and 726, which may unmask the nuclear recognition sequence of β-adducin from the known association of the MARCKS domain of β-adducin with calmodulin (46), and the displacement of calmodulin when serines 713 and 726 of β-adducin are phosphorylated by PKC.

The function of β-adducin in the nucleus and in centrioles of dividing cells remains to be determined, but suggests the possibility that β-adducin may stabilize chromatin and centriolar structure. Thus, phosphorylation of serines 713 and 726 in β-adducin may be a critical step in destabilization of these structures at critical times in the cell cycles. In this context, indirect support for this possibility was found in human U87-MG glioblastoma cells into which a dominant negative *Ptn* gene was introduced; these cells have a high degree of anaploidy and tetraploidy (Y. Chang and T.F.D., unpublished data), raising the possibility that the failure of PTN-dependent phosphorylation of serines 713 and 726 in β-adducin may have reduced the levels of β-adducin phosphorylated at serines 713 and 726 needed to destabilize critical structures associated with nuclear-chromatin and centrioles for chromosomal separation. In Fig. 4, β-adducin appears to be localized to chromosomes during metaphase, supporting the hypothesis that β-adducin and phosphoserine 713 and 726 β-adducin may act to critically regulate stabilization of chromatin and chromosomal separation.

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Identification, cloning, and expression of human estrogen receptor-α36, a novel variant of human estrogen receptor-α66 ☆,☆☆

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Abstract

The identification and subsequent cloning of the 66-kDa human estrogen receptor (here termed hER-α66), its 46-kDa splice variant hER-α46, and the closely related hER-β have had a profound impact on the generation of new understanding of estrogen-mediated functions and led to progress in diagnosis and treatment of human breast cancer. However, a persistent problem has been that not all findings previously reported in estrogen-stimulated cell proliferation can be explained through the known properties of the different estrogen receptors described. As the consequence of a search for alternative mechanisms to account for these different findings, we have now identified, cloned, and expressed in HEK 293 cells a previously unrecognized 36-kDa variant of hER-α66, termed hER-α36. hER-α36 differs from hER-α66 since it lacks both transcriptional activation domains (AF-1 and AF-2) but it retains the DNA-binding domain, and partial dimerization and ligand-binding domains of hER-α66. It also contains three myristoylation sites postulated to direct ER-α36 to the plasma membrane. It is concluded that ER-α36 is a unique variant of ER-α66; ER-α36 is predicted to function as a dominant-negative effector of hER-α66-mediated estrogen-responsive gene pathways and has the potential to trigger membrane-initiated mitogenic estrogen signaling.

Keywords: Estrogen receptor; Breast cancer; Myristoylation sites

The diverse physiological functions of estrogens are mediated by specific nuclear receptors designated as estrogen receptor hER-α66, its splice variant hER-α46, and, the closely related estrogen receptor, hER-β. ERs are defined as ligand-activated transcription factors that regulate transcription of estrogen-responsive genes in the cell nucleus [1–3]. Activation of growth-promoting gene expression in response to 17β-estradiol (E2β), by ERs, especially by ER-α66, is believed to be responsible for estrogen-stimulated cell proliferation.

hER-α66 and hER-β share a common structural architecture [1,2]; both are composed of three independent but interacting functional domains: the N-terminal A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain. The N-terminal domain of hER-α66 harbors a ligand-independent activation function (AF-1), a region that is involved in interactions with co-activators and transcriptional activation of target genes. The DNA-binding domain or C domain contains two zinc-finger-like structures which play important roles in receptor

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☆☆ *Abbreviations:* ER, estrogen receptor; AF, activation function; E2β, 17β-estradiol; E2α, 17α-estradiol; HEK 293 cells, human embryonic kidney 293 cells; NO, nitric oxide; eNOS, endothelial NO synthase.
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dimerization and binding to specific DNA sequences. The C-terminal E/F domain is a ligand-binding domain that mediates ligand binding, receptor dimerization, nuclear localization, and a ligand-dependent transactivation function (AF-2). hER-α46 lacks the first 173 amino acids (A/B or AF-1 domain) due to alternative splicing of the hER-α66 gene that is the result of skipping exon 1 [4]. The alternative splicing event generates an mRNA that has an AUG in a favorable Kozak sequence for translation initiation in-frame with the remainder of the original open-reading frame [4]. hER-β has 96% amino acid identity with hER-α66 in the DNA-binding domain but, in the ligand-binding domain, it is 53% identical to hER-α66; thus, the specificity of ligand binding to these two hERs is significantly different. Even less conservation of amino acid sequence is found between hER-α66 and hER-β in the N-terminal AF-1 and C-terminal domains [1].

A consistent challenge to understanding ER-mediated estrogen-signaling responses has been the role and mechanism of membrane-initiated estrogen signaling. In 1977, Pietras and Szego [5] reported that E2β bound to a cell surface receptor and induced rapid generation of cAMP. Subsequently, many laboratories reported the existence of a plasma membrane-based estrogen receptor that can trigger rapid membrane-initiated estrogen signaling [6–9]. This signaling pathway (also known as a ''non-classic,'' ''non-genomic,'' or ''membrane signaling'' pathway) involves activation of cytoplasmic signaling proteins as well as other membrane-initiated signaling pathways [6–9], including the adenylate cyclase pathway [10], the phospholipase C pathway [11], G-protein-coupled receptor-activated pathways [12], and the MAPK pathway [12–15]. Limited amounts of hER-α66 and hER-α46 have been found to co-purify with 5′-nucleotidase, a plasma membrane marker [14]. In other studies, hER-α46 has been found in association with the plasma membrane, in the cytosol, and in the nucleus of endothelial cells, and it has been found to mediate rapid estrogen-signaled stimulation of NO synthesis [16], suggesting that the hER-α46 isoform also may function as a membrane-based estrogen receptor. Data from different laboratories using the membrane-impermeable compound estradiol-bovine serum albumin (E2-BSA) have indicated the existence of two functionally distinct membrane-associated pathways; one of which is sensitive to anti-estrogens and the other of which is resistant to anti-estrogens [6,7]. For example, in ER-α66 knock-out mice, it was found that there was retention of rapid estrogen-stimulated membrane effects in neurons which were not blocked by ICI-182, 780, a potent estrogen antagonist [17]. While these data made clear that properties of membrane-initiated estrogen signaling exist, the data also indicate that membrane-based ER-α66 and ER-α46 are not sufficient to account for the observed heterogeneity of estrogen effects. Furthermore, the identity of the membrane-based estrogen receptor that mediates these rapid estrogen effects has not been defined.

In this manuscript, we report the identification and cloning of a 36-kDa novel isoform of hER-α66 which we have named hER-α36, to distinguish it from the 66-kDa ER-α (ER-α66) and 46-kDa variant (ER-α46). hER-α36 is generated from a promoter located in the first intron of the hER-α66 gene and differs from hER-α66 since it lacks both of the two transcriptional activation domains (AF-1 and AF-2). However, it retains the DNA-binding domain, and partial dimerization and ligand-binding domains. It has three potential myristoylation sites and thus ER-α36 has the possibility to localize to the plasma membrane. Our data suggest that it may contribute to some of the disparate data about estrogen signaling and contribute in part to estrogen-stimulated development of human breast cancer.

Materials and methods

Homology search and cloning of ER-α36. The sequence homology searches were performed with Blastn and Blastp programs of National Center for Biotechnology Information (NCBI) and used to look for short, nearly exact matches using DNA sequences (20–50 bp in length) encoding the ligand-binding domain of ER-α66. We found one full-length cDNA clone (GenBank Accession No. BX640939) from a human uterus cDNA library that was matched exactly to a DNA sequence encoding the ligand-binding domain of hER-α66 gene. Using the full-length cDNA BX640939 to perform a Blastn search, we found that this cDNA is localized in the human genomic DNA sequence of clone RP1-130E4 on chromosome 6q24.2-25.3 (GenBank Accession No. AL078582). All further sequence alignments were done using the Blast 2 sequence program.

To obtain the open-reading frame of the hER-α36 gene, we used the Marathon Ready cDNA prepared from human placenta mRNA (BD clonetech) as a template for PCR performed according to the manufacturer's recommendation. The PCR primer pairs were designed according to the cDNA sequence of BX640939. The 5′ primer is 5′-CGGAATT CCGAAGGGAAGTATGGCTATGGAATCC-3′ with an *Eco*RI site at the end, and the 3′ primer is 5′-CGGGATCCAGAGGCTTTAGACACG AGGAAAC-3′ with a *Bam*HI site at the end. The cDNA fragment obtained was purified, digested with *Eco*RI and *Bam*HI, cloned into the pBluescript vector to generate pBS-hER-α36, and fully sequenced by the Creighton University Molecular Core Facility.

Cell culture and DNA transfection. Human embryonic kidney (HEK) 293 cells and MCF-7 cells were obtained from ATCC. All cells were maintained at 37 °C in a 5% CO_2 atmosphere in appropriate cell culture media. For DNA transfections, HEK 293 cells were plated at a density of 1×10^5 cells per 60-mm dish and transfected 24 h later with a hER-α36 expression vector driven by the cytomegalovirus (CMV) promoter using the FuGene6 transfection reagent (Roche Molecular Biochemicals). The hER-α36 expression vector was constructed by cloning a 1.1-kb *Eco*RI–*Bam*HI cDNA fragment of hER-α36 from pBS-hER-α36 into the *Eco*RI and *Bam*HI sites of the mammalian expression vector pCB6+. The expression vectors to encode hER-α66 and hER-α46 were obtained from Dr. Nawaz Zafar at the University of Miami.

Western blot analysis and antibodies. For Western blot analysis, cells were disrupted with RIPA buffer, boiled in gel-loading buffer, and separated on a 10% SDS–PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore). The filter was probed with the rat anti-hER-α66 antibody (H222, Research Diagnostic) and visualized with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and the ECL reagents (Perkin-Elmer Life Sciences).

Results

Identification of a novel isoform of hER-α66

Previous reports indicated that three predominant bands of 35–39, 46, and 66 kDa were present in Western

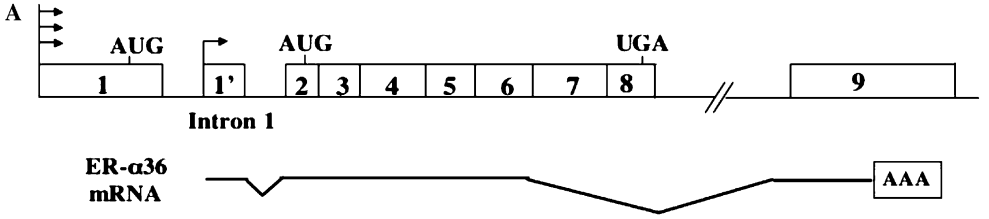
blots probed with the anti-ER-α antibodies raised against the ligand-binding domain of hER-α66 [16,18]. Although these and other reports consistently noted a predominant band at 35–39 kDa, there were no reports that described what this protein band represented. In confirmation, we observed three protein bands (36, 46, and 66 kDa) in Western blot analysis of ER-positive MCF7 breast cancer cells (Fig. 2B) using rat anti-hER-α66 antibodies (clone H222) that recognize the ligand-binding domain of hER-α66. These data, coupled with the previous cloning of the 46-kDa ER-α variant that lacks the first coding exon of the hER-α gene [4], suggested the potential importance of this apparent 36-kDa variant and led us to seek the cDNA that encodes the apparent 36-kDa variant of hER-α.

Through an extensive homology search in GenBank, we identified a full-length clone from a normal human endometrium cDNA library (GenBank Accession No. BX640939) that contains a 5.4 kb cDNA. This cDNA clone encodes a 310 amino acid open-reading frame and a protein with a predicted molecular weight of 35.7 kDa (Fig. 1B). The cDNA sequence of the open-reading frame matches 100% the DNA sequence of exons 2–6 of the hER-α66 genomic sequence. The 5′untranslated region of the cDNA is 100% identical with the DNA sequence of the first intron of the hER-α66 gene. Thus, transcription of this hER-α variant is initiated from a previously unidentified promoter in the first intron of the hER-α66 gene. A small, non-coding novel exon from the first intron of the hER-α66 gene was found and designated as "exon 1," to distinguish it from the original exon 1 in the hER-α66 gene (Fig. 1A). The non-coding exon 1 is directly spliced into exon 2 of the hER-α66 gene and continues from exon 2 to exon 6 of the hER-α66 gene. Exon 6 is spliced to an exon located 64,141 bp downstream of the hER-α66 gene. The genomic organization of the hER-α36 gene is depicted schematically in Fig. 1A. The cDNA sequence encoding the C-terminal 27 amino acids and the 4293 bp 3′untranslated region matched 100% that of a continuous sequence from a genomic clone RP1-1304 on chromosome 6q24.2-25.3 (GenBank Accession No. AL78582). This result indicates that the remaining cDNA sequence of this novel hER-α variant is transcribed from one exon located 4374 bp downstream of the hER-α66 genomic sequence. This exon is thus designated as exon 9, to reflect the additional exon beyond the previously reported eight exons for the hER-α 66 gene (Fig. 1A). These identified splicing events occur at sites of appropriate splice donors and acceptors at the splice junctions.

The predicted hER-α36 protein is initiated from a favorable Kozak sequence located in the second exon, the same initiation codon used to produce hER-α46 [4]. The hER-α36 we have identified differs from hER-α66 by lacking both transcriptional activation domains (AF-1 and AF-2). It retains the DNA-binding domain, and partial dimerization and ligand-binding domains. It possesses a unique 27 amino acid domain that replaces the last 138 amino acids encoded by exons 7 and 8 of the hER-α66 gene (Figs. 1B and 2A).

Cloning of a novel isoform of hER-α66, ER-α36

The open-reading frame of the hER-α36 gene was obtained by RT-PCR from cDNA prepared from human placenta mRNA. Using the PCR primer pairs designed according to the cDNA sequence in GenBank (see Materials and methods), the PCR product was purified, cloned, and fully sequenced; it is 100% identical with the published cDNA clone in GenBank, indicating that hER-α36 is a naturally occurring isoform of hER-66α.



B
1 MAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNR
61 RKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLW
121 PSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRE
181 LVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRN
241 QGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSILLLNSGISHVEAKKRILNLHPK
301 IFGNKWFPRV

Fig. 1. Novel hER-α isoform, hER-α36. (A) Genomic organization of the human hER-α36 gene. The locations of multiple promoters of hER-α66 gene are shown as arrows. The translation start and stop codons are indicated as AUG and UGA. The common exons are shown as numbered open boxes. The extra exon that is beyond the 8 exons found in the hER-α66 gene is numbered as 9 in the open box. The intron 1 is also shown with the exon 1′ in the open box. The lower panel shows mRNA structure of hER-α36 isoform. (B) The predicted amino acid sequence of the hER-α36 open-reading frame. The last 27 amino acids that are unique to hER-α36 are underlined.

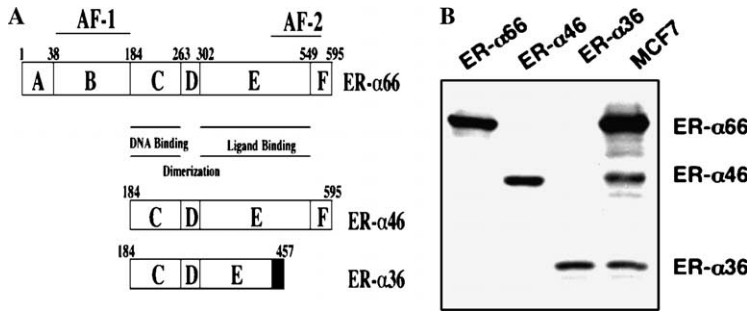


Fig. 2. Domain structure and expression of hERs in HEK 293 cells. (A) Domain structure representation of human hER-α isoforms. Domains (labeled A–F), amino acid sequence numbers, and activation function domains (AF-1 and -2) are shown. The function of each domain is indicated. The last 27 amino acids of hER-α36 are indicated as a filled box. (B) Western blot analysis of three hER-α isoforms. Separate cultures of HEK 293 cells were transfected with expression plasmids containing the different ER-α variants. After 2 days, the cells were lysed and lysates of each transfectant were immunodetected with an anti-hER-α66 antibody (H222) after electrophoresis. Cell extracts from MCF7 cells are used as a positive control.

The hER-α36 cDNA encodes a 36-kDa protein recognized by antibodies directed against the ligand-binding domain of hER-α66

To test whether the cloned cDNA encodes the hER-α36 protein, we performed transient transfection assays in human embryonic kidney (HEK) 293 cells using expression vectors to encode hERs-α66, α46, and α36, respectively. Whole cell extracts from human embryonic kidney (HEK) 293 cells transfected with these expression vectors and non-transfected MCF7 cells were subjected to Western blot analysis with the monoclonal antibody H222 raised against the ligand-binding domain of hER-α66. The results show that a protein with a molecular weight of ~36 kDa was recognized by the H222 antibody in hER-α36 expression vector transfected (HEK) 293 cells (Fig. 2B), but not in cells transfected with an empty vector (data not shown), indicating that the open-reading frame we cloned encodes a 36-kDa protein that can be recognized by the anti-hER-α66 antibodies.

Discussion

In this study, a novel variant of hER-α66 termed hER-α36 has been identified and cloned; the cDNA that was cloned expresses the predicted 36-kDa protein in HEK 293 cells; Western blot analysis of lysates prepared from HEK 293 cells expressing the cloned cDNA revealed that the hER-α36 protein was recognized by an antibody raised against an epitope common to hER-α36, hER-α46, and hERα-66. The novel hER-α isoform is the product of a transcript initiated from a previously unidentified promoter in the first intron of the hER-α66 gene. The hER-α36 protein is identical to regions of the ER-α66 protein encoded by exons 2–6 of the hER-α66 gene. It is devoid of the domains previously identified to have transactivational activities, AF-1 and -2.

The findings we have obtained in this study predict that this unique hER will function as a dominant-negative inhibitor of estrogen-signaled functional responses of hER-α66 through the AF-1 and AF-2 transcription activation domains by virtue of its capacity to bind to the same DNA sequence through the retained DNA-binding domain, but it lacks both AF-1 and AF-2 transcriptional activation domains in hER-α66. This prediction is strengthened since, in a previous report, it was shown that hER-α46, that lacks the AF-1 domain function, is a powerful competitor that acts to suppress the AF-1 activity of hER-α66 [14]. Our data thus suggest the hypothesis that hERα-36 may inhibit functional responses initiated through the transcriptional activation of estrogen-responsive genes by ER-α66 and thus may be an important factor in the development of estrogen-resistant breast cancers.

As cited earlier, the presence of a plasma membrane-based hER that triggers rapid estrogen signaling has been controversial, since the molecular identity of the receptor has not been established nor have the estrogen-signaled functional responses been consistent with each other in different reported studies. In the study of Razandi et al. [12], when either hER-α66 or hER-β was transfected into cells, it was found that these ERs initiated membrane estrogen signaling, although only a very small percentage of either hER-α or ER-β were expressed on the cell surface, suggesting that both hER-α66 and hER-β may be involved in membrane-initiated estrogen signaling, in addition to their traditional roles in genomic estrogen signaling. Furthermore, recently, the 46-kDa isoform of ER-α was found to be localized on the cell surface and to mediate estrogen-stimulated eNOS activation [16].

In these studies we identified three potential myristoylation sites in hER-α36 at residues 25–30 (GVWSCE), 76–81 (GMMKGG), and 171–176 (ELLTNL), raising the possibility that hER-α36 through the ability of the myristoyl residues to localize hER-α36 to plasma membrane sites may also in part initiate estrogen-dependent signaling at the plasma membrane. This possibility is strengthened, since the antibody we generated to the C-terminal 20 amino acids of the predicted hER-α36 recognized a protein associated with the plasma membrane of (HEK) 293 cells transfected with the expression vectors to encode hER-α36 but

not in these cells transfected with the empty expression vector (data not shown). In this context, it may be important to note that the three putative myristoylation sites in hER-α36 and hER-α46 are localized proximal to the N-terminus, a localization that favors myristoylation, whereas the putative myristoylation sites in hER-α66 are found further away from the N-terminus of ER-α66. It is thus possible that hER-α36 and hER-α46 are both well positioned to initiate membrane-initiated estrogen signaling. It is suggested that hER-α36 has the potential to initiate membrane estrogen signaling, although the evidence to date is based on computer-based analysis of the cloned cDNA. Since ER-α36 totally lacks both transactivation domains and thus may only function as an inhibitor of genomic estrogen signaling, ER-α36 may principally function as a membrane-based estrogen receptor to mediate membrane-initiated estrogen signaling.

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Identification of the angiogenesis signaling domain in pleiotrophin defines a mechanism of the angiogenic switch ☆

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Abstract

Neoplasms progress through genetic and epigenetic mutations that deregulate pathways in the malignant cell that stimulate more aggressive growth of the malignant cell itself and/or remodel the tumor microenvironment to support the developing tumor mass. The appearance of new blood vessels in malignant tumors is known as the ''angiogenic switch.'' The angiogenic switch triggers a stage of rapid tumor growth supported by extensive tumor angiogenesis and a more aggressive tumor phenotype and its onset is a poor prognostic indicator for host survival. Identification of the factors that stimulate the angiogenic switch thus is of high importance. Pleiotrophin (PTN the protein, *Ptn* the gene) is an angiogenic factor and the *Ptn* gene has been found to be constitutively expressed in many human tumors of different cell types. These studies use a nude mouse model to test if *Ptn* constitutively expressed in premalignant cells is sufficient to trigger an angiogenic switch in vivo. We introduced an ectopic *Ptn* gene into ''premalignant'' SW-13 cells and analyzed the phenotype of SW-13 *Ptn* cell tumor implants in the flanks of nude mice. SW-13 *Ptn* cell subcutaneous tumor implants grew very rapidly and had a striking increase in the density of new blood vessels compared to the SW-13 cell tumor implants, suggesting that constitutive PTN signaling in the premalignant SW-13 cell implants in the nude mouse recapitulates fully the angiogenic switch. It was found also that ectopic expression of the C-terminal domain of PTN in SW-13 cell implants was equally effective in initiating an angiogenic switch as the full-length PTN whereas implants of SW-13 cells in nude mice that express the N-terminal domain of PTN grew rapidly but failed to develop tumor angiogenesis. The data suggest the possibility that mutations that activate *Ptn* in premalignant cells are sufficient to stimulate an angiogenic switch in vivo and, since these mutations are frequently found in human malignancies, that constitutive PTN signaling may be an important contributor to progression of human tumors. The data also suggest that the C-terminal and the N-terminal domains of PTN equally initiate switches in premalignant cells to cells of a more aggressive tumor phenotype but the separate domains of PTN signal different mechanisms and perhaps signal through activation of a separate receptor-like protein.

Keywords: Pleiotrophin; Angiogenic switch; Angiotensin II; RPTPβ/ζ

Pre-malignant cells progress to become more malignant through sequential genetic and epigenetic mutations in individual malignant cells that initiate ''switches'' in signaling pathways to convert the phenotype of the malignant cell to one of higher malignancy [1–3]. The onset of tumor angiogenesis, known as the angiogenic switch [3,4], is essential for nutrients, for adequate oxygenation of the growing tumor mass, and for entry sites for tumors to metastasize to distant organs [5–7]; it is an essential event for premalignant cells to exit dormancy. Deregulation of different factors that are inducers or inhibitors of

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angiogenesis has been postulated to be mechanisms to initiate the "angiogenic switch." The angiogenic factors basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF) are expressed in many different human cancers and thus have been viewed as important components of the angiogenic switch [8–10]. However, neither bFGF nor VEGF alone appears to be sufficient for the full picture of the angiogenic switch and thus the pursuit of different regulators of angiogenesis and models to investigate their putative roles in stimulating the angiogenic switch is important not only to better understand tumor progression but to design new therapies to treat aggressive malignancies.

Pleiotrophin (PTN the protein, *Ptn* the gene) is a recently identified and characterized cytokine with established roles in normal and transformed cell growth. It is also an angiogenic factor capable of stimulating new capillary and arteriolar growth in injured tissues [11,12]. Pleiotrophin is a secreted protein of 136 amino acids with lysine rich domains at the N- and C-termini [13] and two separate heparin-binding thrombospondin type 1 repeat domains linked by a short amino acids sequence internally [14]. Pleiotrophin is a highly conserved cytokine [13,15]; it signals proliferation of different cells in culture [13,15] and induces lineage specific differentiation of different progenitor cells [15–17]. The *Ptn* gene also is a protooncogene [18] and expressed in many different malignant cells [13,15,19,20]. The highly malignant phenotype of different malignant cells reverts to the phenotype of the pre-malignant cell when a dominant negative mutant *Ptn* gene [20] is introduced, suggesting that PTN-dependent signaling pathways are responsible for the highly malignant phenotype of aggressive cancers in which the *Ptn* gene is expressed. Furthermore, PTN-dependent signaling pathways appear to stimulate tumor angiogenesis, since, in other experiments, it has been found that the angiogenic phenotype of glioblastoma cell tumor implants in nude mice is entirely reversed when the dominant negative *Ptn* gene was introduced into them, suggesting that PTN in human glioblastoma cells is sufficient alone to initiate the angiogenic switch. These studies collectively suggest that mutations that trigger constitutive expression of the *Ptn* gene in premalignant cells deregulate pathways that provide selective growth advantage, and thus favor selection of clones of *Ptn* expressing malignant cells in the growing tumor mass. In support of this suggestion, *Ptn* expression is found with high frequency in many highly malignant cancer cells [13,15,19,20], perhaps as the result of clonal selection of the cells that express *Ptn* during tumor progression.

These studies were designed to test whether activation of the endogenous *Ptn* gene is sufficient in the premalignant cell to initiate an angiogenic switch. To test this hypothesis, premalignant SW-13 cells into which an activated *Ptn* gene was introduced were implanted in flanks of nude mice and examined for growth and tumor angiogenesis. We also tested the separate N- and C-terminal domains of PTN to attempt to identify which domain in PTN may initiate the different phenotypes in premalignant cells that express the endogenous *Ptn*.

Materials and methods

Expression constructs. The full-length human *Ptn* cDNA (GenBank Accession No. NM_002825) encodes a 168 amino acid protein that includes a 32 amino acid signal peptide. The cDNAs encoding the N-terminal (amino acid residues 1–64) and C-terminal (amino acid residues 69–136) domains of PTN and the full-length PTN (amino acid residues 1–136) were coupled with the endogenous signal peptide and cloned into the PAGE 103 vector as previously described [21].

Cells, cell culture, and DNA transfection. Human adrenal carcinoma (SW-13) was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. DNA transfections were performed by calcium phosphate co-precipitation as described [18]. The transfectants were selected with G418 for three weeks. In each case, colonies were clonally selected, and the clonal cell lines were established, expanded, and confirmed by Northern blot (Fig. 1A) and Western blot (Fig. 1B) analyses. Clones with high-level expression of the exogenous PTN protein or its variants' gene were retained for further study.

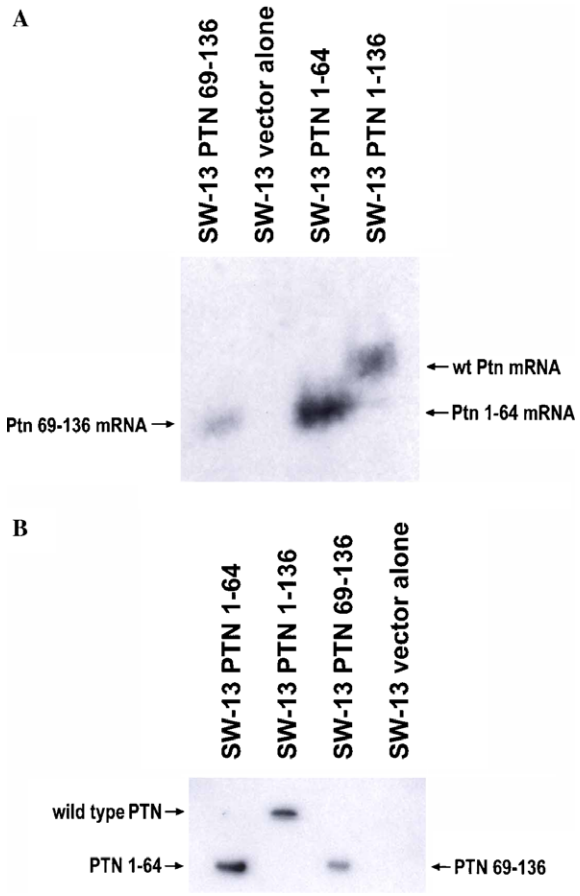

Fig. 1. SW-13 cells with stable expression of an exogenous full-length *Ptn* gene (SW-13 PTN 1–136) or an exogenous truncated *Ptn* gene encoding the N-terminal 1–64 amino acids (SW-13 PTN 1–64) or the C-terminal 69-136 amino acids (SW-13 PTN 69–136) were analyzed in Northern blots (A) and Western blots (B), and found to be expressed in high level; the clones with high level expression of PTN and the truncated PTNs were selected for these experiments.

Northern blots. Total RNA was extracted from cells with Trizol. [α-^{32}P]dCTP labeled-*Ptn* cDNA was used as a probe for overnight hybridization with total cellular RNA. Blots were washed with 0.1× SSC and 0.1% SDS at room temperature and exposed to X-ray film with intensifying screens at −70 °C as previously described [21].

Western blot analysis. Cloned transfectants that harbor the expression vectors described above or an empty vector (control) were lysed in cell lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, and 0.5% NP40) and centrifuged. The supernatants were incubated with heparin–Sepharose beads (Pharmacia) at 4 °C for 24 h, washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.35), and eluted with SDS gel loading buffer. The eluates were separated by 12.5% SDS–PAGE, transferred to Immobilon membranes (Millipore), immunoblotted with chicken anti-human PTN antibodies, and visualized by the ECL system (Amersham).

Transformation, tumor progression, and tumor formation in nude mice. NIH/3T3 cells were used to assay transformation as previously described [21]; the tests used include loss of contact inhibition in monolayer cultures, the ability to form colonies in soft agar, and the ability to form subcutaneous tumors in flanks of nude mice. Female athymic nude mice (6-week-old nu/nu; Harlan Sprague–Dawley, Indianapolis, IN) were injected subcutaneously in each flank with 2×10^6 cells from pooled clonal cell lines. Tumor size was checked daily, beginning 10 days after injection. At 6 weeks, selected animals were sacrificed and tumor size was estimated as the product of two diameters at right angles to each other [18]. Representative tumors from each of the established cell lines were examined by hematoxylin and eosin (H and E) staining, and analyzed for intratumor microvessel density (IMD) [22]. Intratumor microvessel density is a measure of the relative density of tumor angiogenesis in sections of tumor tissue stained with anti-CD31 (PECAM-1) monoclonal antibodies. Stained capillaries were counted at 400× magnification by independent, uninformed investigators and statistically analyzed using Student's t test.

Results

Ectopic expression of pleiotrophin and its N- and C-terminal domains in pre-malignant SW-13 cells

To test the possibility that constitutive expression of *Ptn* is sufficient to switch the premalignant cell to a highly malignant cell in vivo [21,23], SW-13 cells were transfected with expression constructs to encode PTN residues 1–136 (full length), 1–64 (N-terminal), 69–136 (C-terminal), and an "empty vector" (for use as control). SW-13 cells are derived from a human adrenal carcinoma; SW-13 cells grow slowly in culture, form few colonies when grown in soft-agar, and fail to effectively grow as tumor implants in flanks of nude mice, suggesting that the SW-13 cells may be ideal premalignant cells to test the model that ectopic *Ptn* expression may initiate an angiogenic switch. The full-length PTN was tested to seek whether the full-length PTN is sufficient alone to stimulate a switch to a more aggressive phenotype of SW-13 *Ptn* cells in vivo, and the N-terminal (PTN residues 1–64) and C-terminal (PTN residues 69–136) domains of PTN were tested to see if the separate N- and C-terminal heparin binding, thrombospondin type I domains are able to signal independently of the full-length PTN and to confer a distinct phenotype in SW-13 cells tumor implants. Clonally selected SW-13 cells with highest expression of each vector construct (see Fig. 1) were implanted in the flanks of nude mice and the rate of growth, the size of the tumors at 6 weeks, the histological features of the tumors that developed at these sites, and the numbers of new blood vessels (IMD) formed within the tumor mass were analyzed.

Ectopic expression of pleiotrophin and its C-terminal domain initiates an angiogenic switch in premalignant SW-13 cells

The SW-13 vector-control cells developed only small (12 mm^2), avascular (IMD 5 vessels/field) subcutaneous tumor implants at sites of injection in flanks of nude mice, consistent with the anticipated premalignant phenotype of the SW-13 cell. In contrast, SW-13 PTN 1–136, SW-13 PTN 1–64, and SW-13 PTN 69–136 cell tumor implants each grew rapidly at sites of implantation. The SW-13 PTN 1-136 cell subcutaneous tumor implants grew more rapidly than the SW-13 PTN 1–64 cell subcutaneous tumor implants and were larger (60 mm^2 vs. 44 mm^2) at 6 weeks (Table 1); the intratumor microvessel density (IMD) in SW-13 PTN 1–136 cell subcutaneous tumors was 30 vessels/field whereas the IMD of the SW-13 PTN 1–64 cell subcutaneous tumors was 7 vessels/field, a value little different from the 5 vessels/field IMD of the SW-13 empty vector control cells (Table 1). The SW-13 PTN 69–136 cell tumor implants were the most rapidly growing and were the largest of the tumors that developed at 6 weeks (85 mm^2, Table 1); they also had the highest IMD (∼45 vessels/field, Table 1) in sections stained with anti-CD31 antibodies (Fig. 2) of any of the SW-13 cell PTN variants studied. Thus, SW-13 PTN 1–136 cell, SW-13 PTN 1–64 cell, and SW-13 PTN 69–136 cell tumor implants each grew rapidly as subcutaneous tumors; constitutive expression of PTN 1–136, PTN 1–64, and PTN 69–136 initiates a switch of the premalignant cell to a cell with significantly more malignant phenotype. However, only the SW-13 PTN

Table 1
Tumor formation and intratumor microvessel density

	Tumor (mm^2)	IMD (#/field)
Vector alone	12 ± 2	5 ± 0.2
PTN 1–136	$60 \pm 5^{*,b}$	$30 \pm 4.3^{*,b}$
PTN 1–64	$44 \pm 3^{*}$	7 ± 2.6
PTN 69–136	$85 \pm 7^{*,b,a}$	$45 \pm 5.4^{*,b,a}$

Tumor formation and intratumor microvessel density (IMD) in tumor implants in athymic nude mice at sites of implantation of cloned SW-13 cell line transfected with full-length *Ptn*, its mutant gene constructs, or PAGE 103 vector alone. Four animals per group were injected with 2×10^6 cells/site. Tumors were measured 6 weeks after the injection of cells. The tumor size (mm^2) was calculated from the product of perpendicular diameters of tumors. Intratumor microvessel density (IMD) was used to evaluate tumor angiogenesis. The tumor sections were immunostained by an anti-mouse CD31 monoclonal antibody using a protocol described in Materials and methods. Stainable capillaries (#) were counted at 400× magnification field by independent, uninformed investigators. The results are shown as average ± SE.

[a] $p < 0.05$ vs. PTN 1–136.
[b] $p < 0.05$ vs. PTN 1–64.
[*] $p < 0.05$ vs. vector alone.

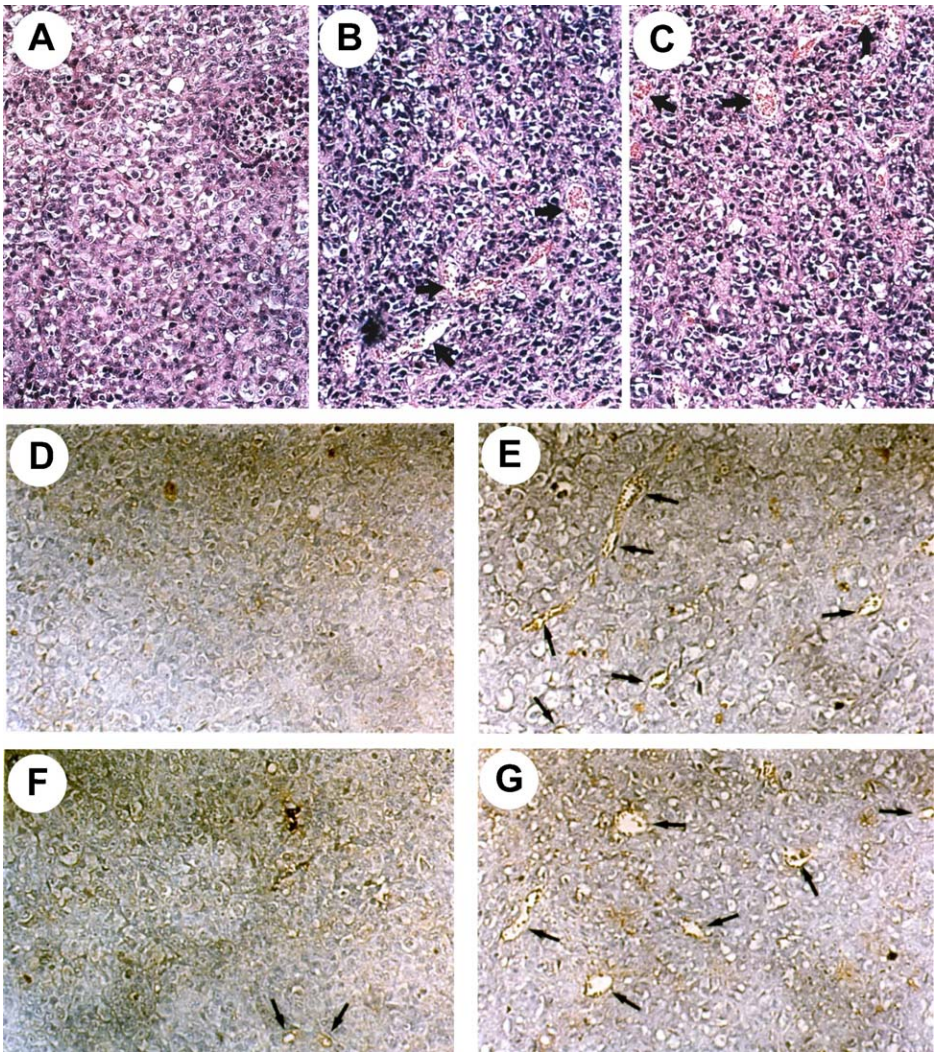

Fig. 2. Hematoxylin and eosin staining of SW-13 PTN tumor sections shows a large number of blood vessels (arrows) in SW-13 PTN 1–136 cell tumors (B) and SW-13 PTN 69–136 tumors (C) cells but not in SW-13 PTN 1–64 cell tumors (A). Immunohistochemistry of tissue sections of the tumor implants formed from SW-13 cells with stable expression of PTN 1–136 or the truncated mutant PTNs in the nude mouse, stained with anti-CD31 monoclonal antibodies (see Materials and methods, above). Arrows indicate blood vessels. The intratumor microvessel density (IMD, see Materials and methods, above) was markedly increased in tumor implants formed from the SW-13 cells expressing exogenous full-length PTN 1–136 (E) and PTN 69–136 (G), but not in tumors formed from the SW-13 cells which expressed PTN 1–64 (F) or harbored vector alone as control (D).

1–136 and the SW-13 PTN 69-136 cell tumors developed the striking increase in numbers of new blood vessels anticipated of an angiogenic switch. The data thus suggest that activated PTN signaling in the premalignant cells is sufficient to stimulate an angiogenic switch in vivo. The data also suggest that the C-terminal domain of PTN is the ''angiogenic domain,'' that the N- and C-terminal domains signal independently of each other, that the N- and C-terminal domains of PTN initiate switches to the more malignant phenotype through different mechanisms, and that the N- and C-terminal domains of PTN likely signal through separate receptor-like domains.

Sections stained with hematoxylin and eosin were used to demonstrate the large number of blood vessels (arrows) in SW-13 PTN 1–136 cell tumor implants (Fig. 2B) and SW-13 69-136 tumor implants (Fig. 2C) cells but not in SW-13 1-64 cell tumor implants (Fig. 2A) nor SW-13 empty vector control cell tumor implants (data not shown). A surprising finding was the larger size of the vessels in SW-13 PTN 1–136 and SW-13 PTN 69–136 cell implants than were found in vessels in SW-13 PTN 1–64 cell tumor implants, suggesting the possibility they may be arterioles. This finding may be significant, since, in another study, PTN was found to initiate both new capillaries and new arteriols in ischemic myocardium injected directly with PTN [12] and to stimulate significantly larger vessels in transgenic mouse tumors with constitutive expression of the *Ptn* gene.

Representative sections stained with anti-CD31 antibodies illustrate the striking differences in numbers of new blood vessels between SW-13 PTN 1-136 (Fig. 2E), SW-13 PTN 1–64 (Fig. 2F), SW-13 PTN 69–136

(Fig. 2G), and SW-13 (vector alone) (Fig. 2D) cell tumors; the SW-13 (vector alone) and SW-13 PTN 1–64 cell subcutaneous tumor implants are seen to be relatively avascular whereas SW-13 PTN 1–136 cell and SW-13 PTN 69-136 cell subcutaneous tumor implants are filled with many tumor-associated vessels of varying size (arrows).

Discussion

The data in this manuscript support the conclusion that constitutive activation of the endogenous *Ptn* gene in premalignant cells may be sufficient to switch the premalignant phenotype to a highly malignant phenotype and thus to favor the selective growth of the *Ptn* expressing cells in the tumor mass. The data suggest that the switch initiated in SW-13 cells by expression of either the full-length PTN 1-136 or PTN 69–136 is an ''angiogenic switch''; it is characterized by exuberant subcutaneous tumor growth and abundant new blood vessel formation. In contrast, whereas the switch initiated by PTN 1–64 also triggers rapid tumor implant growth, the SW-13 PTN 1–64 cell tumor implants contain few new intra-tumor microvessels. We have termed the C-terminal domain of PTN the ''angiogenic domain'' in contrast to the previously termed N-terminal ''transforming'' domain of PTN [21]. Both the N-terminal and the C-terminal domains of PTN contain heparin-binding thrombospondin type I β-sheet repeat domains with significant homology to each other [14]. The extreme N-terminal domain is very lysine-rich as is the extreme C-terminal domain of PTN. The role of these very basic clusters of amino acids in PTN signaling is not known. What the data suggest is that the separate signaling domains of PTN promote tumor progression in premalignant cells by different mechanisms and raise the possibility that the separate independent signaling pathways initiated through the N- and C-terminal domains of PTN signal through separate receptor-like proteins.

The mechanisms through which PTN stimulates angiogenesis remain to be fully unraveled. PTN signals through the Receptor Protein Tyrosine Phosphatase (RPTP)β/ζ [24] and, through the PTN/RPTPβ/ζ signaling pathway, PTN stimulates phosphorylation of different cytoskeletal and other proteins, and induces the phenotypic changes characteristic of an epithelial-mesenchymal transition [25]. PTN also regulates the renin-angiotensin pathway [26], a pathway known to be pro-angiogenic [27], and highly angiogenic tumor implants from glioblastoma with high level expression of *Ptn* no longer are angiogenic when the nude mice are treated with captopril, an inhibitor of the angiotensin converting enzyme, the rate-limiting enzyme in the synthesis of angiotensin II [28]. Furthermore, PTN is directly angiogenic when injected into ischemic myocardium; in this context, PTN stimulates both new capillary formation and new arteriolar formation and the new blood vessels formed establish functional anastamoses with the systemic circulation, indicating that PTN, more than other angiogenic factors studied, stimulates a more completely and more fully developed neovasculature [12]; this finding, as suggested above, may account for the larger vessels seen in the SW-13 PTN 1–136 cell tumor implants.

These studies thus suggest that mutations that trigger constitutive PTN signaling during tumor progression may be the clearest example of the mechanism of an angiogenic switch; they also suggest that PTN signaling may be an important target for therapy of advanced human malignancies.

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